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Oxygen Evolution from Algae Illuminated by Short and Long Flashes of Light

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Received 6 January 1958

SUMMARY

The oxygen produced by illuminating *Ankistrodesmus braunii* with single light flashes has been determined using the Hersch galvanic oxygen cell. Measurements were made with the cells suspended in alkaline solution equilibrated with nitrogen containing oxygen at a partial pressure of 10^{-4} mm. Hg.

1. A single light flash, if very brief (less than 5 millisec.) results in no measurable oxygen production; a longer flash (35 millisec.) gave a yield of approximately 1 mole O_2/800 moles chlorophyll.

2. A pair of flashes suitably spaced gave a greater yield than the sum of the yields when given individually, although one was so brief that by itself it produced no measurable oxygen. The yield of a long flash preceded by a short flash was twice as great as that of the long flash given alone; when the flash order was reversed the combined yield was smaller but still greater than for the long flash alone.

3. The combined yield of a pair of flashes varies with the interval separating the flashes, rapidly rising to a maximum and then decaying more slowly. With a long and short flash the optimal interval was 0·7 sec. but some enhancement of yield was observed when the flashes were separated by as long as 10 or 15 sec.

4. When the flashes were superimposed on background illumination the yields were increased and were measurable even for the short flashes. Measured with background illumination the optimal yield for a pair of short flashes was obtained with flashes separated by about 0·05 sec.

INTRODUCTION

MOST previous studies of algal photosynthesis in intermittent light have been confined to measurements of the average integrated rate of net oxygen production during a large number of light flashes and intervening dark periods with a total duration of many minutes. With the development of especially sensitive methods of assay for oxygen, measurement of photosynthesis resulting from a single flash became possible. Whereas studies in steady state flashing-light experiments were confined to the repetition of a single flash type, further combinations involving both short and long flashes may be explored when yields for individual flashes are measured.

Allen and Franck (1955) using the phosphorescence quenching method of

[1] This work was completed whilst one of us (A. H. B.) was a fellow of the John Simon Guggenheim Foundation.

Pringsheim (Pollack, Pringsheim, and Terwood, 1944) measured yields for a single flash or pairs of flashes. A single flash of less than a millisecond in duration resulted in no detectable oxygen evolution from *Scenedesmus*; flashes of longer duration gave measurable yields. When measurements were made with pairs of flashes they found that some effect of the first flash carried over through the intervening dark interval since the yield resulting from two closely spaced flashes was greater than the sum of the yields of the flashes separated by a very long dark interval. They also observed that the yield per flash was increased when the flash was superimposed on a continuous background light of low intensity.

The present work using a different organism and different methods confirms and extends the observations of Allen and Franck.

MATERIALS AND METHODS

The organism used was the green alga *Ankistrodesmus braunii* (Naegeli) Brunnthaler. A pure culture, obtained from Dr. R. E. Norris, was grown autotrophically in medium V described by him (Norris *et al.* 1955). Flasks were illuminated continuously from below by a bank of 4×15 W fluorescent lamps providing an incident intensity of about 350 f.c. Growth was at 24°–27° C. and a 5 per cent. CO_2/air mixture bubbled continuously through the cultures. After 3 to 4 days' growth (when the culture density was about 2 μl./ml.) cells were harvested by centrifuging at 1,000 *g*. They were washed once in glass distilled water and resuspended in M/15 $KHCO_3$. Packed cell volumes were measured and in addition chlorophyll determinations were made. Between 30 and 80 μl. cells in 4 ml. suspension were used in the experimental vessel. Measurements of oxygen production were at a temperature between 18° and 22° C.

Long flashes were generated by Phillips Photoflux photographers' flash bulbs and brief flashes from Mazda xenon discharge tubes. The tubes were fired by condenser discharge and were triggered with tesla coils. Flash bulbs B_{38}, B_{45}, B_{60}, B_{100} differing in intensity and duration were used. The intensity range could be increased further by firing two bulbs simultaneously. From a comparison of the yields obtained it was demonstrated that flash saturation was achieved using any of the bulbs with the possible exception of B_{38} (which had the lowest peak intensity and shortest duration). In all the experiments described below the single-bulb type B_{60} was used. The nominal peak intensity was $2{\cdot}8 \times 16^6$ lumens. A small proportion of the bulbs were unusual in producing a prolonged light output; these 'flares' could be detected visually and the results were discarded. The remainder gave a closely uniform output as judged by the constancy of the photosynthetic yield.

Discharge tubes, Mazda FA4 and FA15—designated by us E_4 and E_{15}—gave flashes of approximately 0·2 and 5 milliseconds duration respectively (see Fig. 1). Each provided a peak intensity of approximately 10^6 lumens (assuming 40 lumens/watt). Reduction of the storage capacitor to one-half

made no change in the yields observed indicating that the flashes were 'saturating'. The flash tubes were mounted a few cm. and the flash bulbs 15 cm. from the experimental vessel. Timing of flashes was by cam-activated micro-switches so that, by relative angular positioning of two cams on the same shaft and by control of the speed of shaft rotation, a wide range of flash separations could be investigated.

When background illumination was used, this was provided by a mercury lamp with UV, infra-red, blue and neutral filters. The light was chiefly but not exclusively from the 405 mμ Hg line and the intensity was such that it gave a net photosynthetic rate about 0·1 per cent. that of 'saturation'.

Oxygen was determined by means of a flow system whose construction has been described previously (Whittingham, 1956). It consisted of components for purifying a nitrogen stream, a cylindrical experimental vessel, a caustic potash scrubber and a Hersch oxygen cell. The oxygen was determined amperometrically from the output of the Hersch cell which was amplified and recorded on a Brown recording potentiometer. The flow was maintained constant within an experiment at a value between 2 and 3·5 l./hr. and the total volume between the experimental vessel and the Hersch cell was about 130 ml. Figs. 2 and 4 show tracings of the recorded chart when oxygen was produced as a result of light flashes. Calibration was effected by introducing into the gas steam known volumes of gas mixtures. In an earlier paper (Whittingham, 1956) calibration had depended on the production of oxygen electrochemically assuming Faraday's Law. This was found to be unreliable when very small electrolysis currents were used and the electrochemical calibration has been retained for relative determinations only.

The yield of oxygen can be determined from the product of the area under the recorded curve (after suitable correction for the base line) and the flow rate. With constant flow this area was found to be proportional to the maximum chart pen displacement and this measure was frequently used making it unnecessary to make measurements with a planimeter of all chart records.

The partial pressure of oxygen in the gas stream when equilibrated with the algal suspension was of the order of 10^{-4} mm. Hg, approximately one order of magnitude higher than prevailed in the experiments of Allen and Franck. All effects reported here were observed over a 5-fold range of O_2 partial pressures and were independent of the absolute partial pressure. After insertion in the apparatus the cell suspension became progressively more anaerobic during the first 2 hours and remained at a partial pressure of 10^{-4} mm. Hg for 2 to 4 hours of experimentation. The organism chosen was relatively insensitive to protracted anaerobiosis as compared with *Chlorella* or *Scenedesmus*; this was one reason for the choice of this alga. After as long as 6 hours at concentrations of oxygen between 1 and 10 v.p.m. the time course of photosynthetic induction measured in continuous illumination was not measurably prolonged compared with that following an aerobic dark period of 5 minutes. Even after 12 hours anaerobiosis the light 'saturated' photosynthetic rate was unchanged.

RESULTS

No oxygen production was detectable from a single brief flash (E_4 or E_{15}). A long flash (B_{60}) gave an oxygen yield of approximately 1 mole O_2/800 moles chlorophyll. When a brief flash preceded a long flash the combined yield

FIG. 1

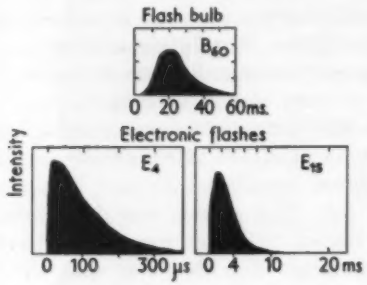
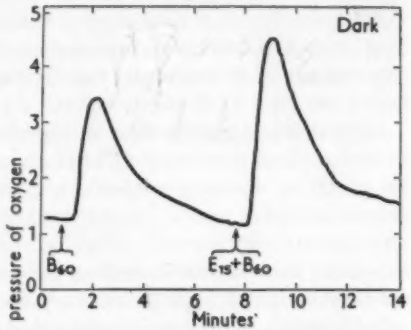


FIG. 2



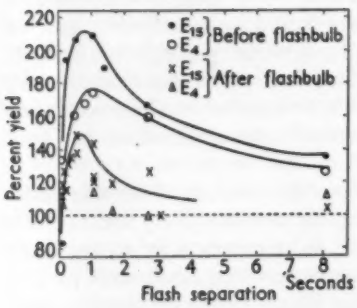


FIG. 3

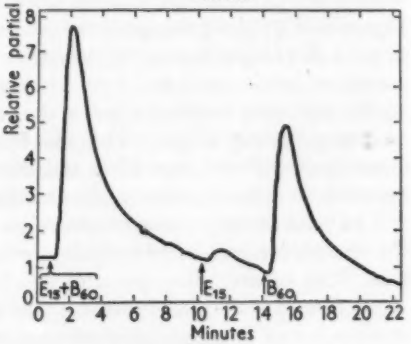


FIG. 4

FIG. 1. The three types of flash used. The curves show light intensity as a function of time, the flashbulb from data given by the manufacturers and the electronic flashes as measured by us. The ordinate is in arbitrary units in each case.

FIG. 2. A recorder tracing showing the response to a flashbulb and to the flashbulb preceded by an electronic flash.

FIG. 3. The combined yield obtained from a flashbulb preceded by an electronic flash (E_4 ○ or E_{15} ●) or following an electronic flash (E_4 Δ or E_{15} ×) plotted against the time interval between the flashes. The ordinate is the yield for the pair of flashes relative to the yield of the flashbulb. The electronic flashes (E_4 and E_{15}) give no measurable yield by themselves.

FIG. 4. A recorder tracing for a suspension illuminated by dim background light showing the response to a flashbulb, an electronic flash and the two given closely spaced (in reverse order). Under these conditions a measurable yield is obtained from all three types of flash.

was appreciably greater than for a single long flash alone. Fig. 2 shows the result of a representative experiment illustrating the yield for a long flash with and without prior excitation of the algae by a brief flash. The increase in long-flash yield resulting from pre-excitation with a brief flash depended on

the dark interval between the flashes. Fig. 3 shows that the maximal yield was obtained with a spacing of about 0·7 sec. and that some influence of the short flash was detectable even for dark intervals of 10 sec. or longer. Reversing the order of the flashes—long followed by short—also resulted in an increase in yield but the effect was smaller. Similar results were obtained whether E_4 or E_{15} was used as the pre-exciting flash.

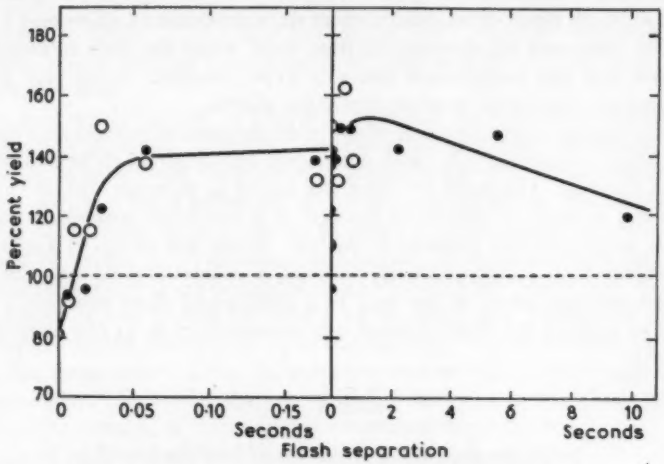


FIG. 5. The yield obtained from two electronic flashes, E_4 preceding E_{15} by the interval shown on the abscissa, in the presence of continuous background illumination. The ordinate is the combined yield as a percentage of the sum of the yields when the flashes are given separated by a very long dark interval.

Both of the brief flashes gave measurable yields when they were given with the algae continuously illuminated with a very low intensity background light. The yield from the long flash (B_{60}) also was greatly increased when the flash was superimposed on background illumination. As the intensity of background light was increased the absolute yields increased but the maximal percentage yield obtained from a pair of flashes compared with the sum of the individual flash yields was decreased (see Table I). Fig. 4 is a recorder tracing of flash effects in the presence of background light and may be compared with Fig. 2 where there was no background illumination.

Since even a brief flash gives measurable oxygen production when superimposed on background illumination it was possible under this condition to investigate the interaction between two brief flashes. The results are shown in Fig. 5 where the yield is plotted as a percentage of the sum of the yields for the flashes given widely spaced. The curve is qualitatively similar to that of Fig. 3 rising quickly to an optimum and then declining gradually. In this case, however, the optimum was attained with a spacing of $5 \cdot 10^{-2}$ sec. instead of 0·7 sec.

When two flashes were given simultaneously (both long or one long and one short) there was a decrease in flash yield to a value less than that of the sum of the individual flash yields. (Compare Figs. 3 and 5.) We have also found that, when the background intensity was high, a flash resulted in subsequent inhibition for some minutes of the photosynthetic activity measured from the background light. In general the intensity used was kept as low as possible and no complications of this type were present in the experiment illustrated in Fig. 5. Both types of inhibitory effect were observed by Allen and Franck who also measured the decrease in flash yield when the flash intensity was increased and the background intensity kept constant. Allen and Franck attributed the inhibition to photo-oxidative effects.

Measurements were also made of the yield obtained with repetitive flashing of the briefest type flash (E_4) with a constant dark interval chosen to be near optimal (1 sec.). The yield *per flash* was found to increase with number of flashes for a group of up to 20 flashes. For technical reasons it was not possible to exceed this number of flashes. When the group of flashes was superimposed on background illumination a maximal yield per flash was obtained with groups of as few as 3 to 4 flashes and there was only a small change in yield as the flash number was increased up to 24 (an insignificant decrease).

TABLE I

Yields per flash for a succession of brief flashes (E_4)

Flash tube FA_4. Flashes separated by 1 sec. periods

	Relative yield					
	Dark		*Weak diffuse light*		*Higher intensity diffuse light*	
No. of flashes	*Total*	*Per flash*	*Total*	*Per flash*	*Total*	*Per flash*
1	0	0	0·29	0·29	(1·10)	(1·10)
3	—	—	1·96	0·65	—	—
5	0·88	0·18	—	—	—	—
6	—	—	4·25	0·71	7·72	1·29
9	—	—	9·58	1·06	10·0	1·11
10	2·84	0·284	—	—	—	—
12	—	—	—	—	12·7	1·06
18	—	—	—	—	18·9	1·05
20	6·54	0·327	—	—	—	—
24	—	—	—	—	24·3	1·01
8	—	—	—	—	8·57	1·07
4	—	—	—	—	4·42	1·11
B_{60}	1·17	—	4·76	—	9·72	—
E_4 B_{60} (max.)	2·55	—	6·00	—	11·30	—
E_4+E_4 effect	1·38	—	1·24	—	1·58	—
E_4 effect	+1·38	—	+0·95	—	+0·48	—

In the measurements at the highest intensity there was some change in yield with time. The column (apart from the value given in brackets) is a time sequence from top to bottom.

DISCUSSION

A single flash of several milliseconds duration resulted in no measurable oxygen production. Repetition of such a flash resulted in a progressive increase in yield with number of flashes until a limiting value was reached. Longer flashes of some 30 milliseconds duration gave a measurable yield, but the yield was greatly increased if the longer flash was preceded by another flash—even although the preceding flash may be so brief as to give no measurable yield by itself. Thus a first flash must either remove something which decreases and/or produce something which increases the production of oxygen by a second flash.

One view would be that the first flash results in the removal of accumulated fermentation products. Inductive effects which have been attributed to this phenomenon have been observed with *Chlorella* and to a smaller extent with *Scenedesmus*, the latter being the organism used by Allen and Franck who found results similar to ours. Such effects if present in *Ankistrodesmus* are not measurable by the usual methods, but cannot be excluded when such small amounts of product as are produced by a single flash are considered. A cumulative effect of two flashes was observed by Allen and Franck not only for photosynthesis but also for the quinone reaction. No measurable induction phase has been observed for the quinone reaction which again suggests that the cumulative effect of a number of flashes may not be explained solely in terms of the removal of accumulated fermentation products.

A second view is that a first flash may by itself be unable to result in oxygen production because some essential catalyst is in an inactive form. If it is postulated that such a catalyst is activated only in light our data show that the greatest effect of a first flash on a second is not attained until 0·7 seconds after the first flash. This suggests that after activation by light the catalyst must undergo further change thermochemically before it can catalyse oxygen production. Since in the dark the catalyst reverts to an inactive form the concentration of active catalyst following the first flash will go through a maximum. Such a mechanism would be in qualitative agreement with our results.

Previous studies with intermittent illumination have been confined to determinations of the average yield per flash from a group of flashes. In general they indicate the existence of at least two velocity constants which determine the storage of light energy. 'Short' flashes of the type used by Emerson and Arnold (1932) gave a maximum yield per flash of about 1 mole O_2/mole chlorophyll with a maximum frequency between flashes for maximal yield of 0·01 sec. (20° C.). When later, flashes of several milliseconds duration were used, i.e. 'long' flashes the yield was greater the longer the flash, was temperature dependent (unlike the Emerson and Arnold yield) and the maximum frequency of flashing for maximum yield of the order of 1 per sec. (Briggs, 1941; Weller and Franck, 1941; Tamiya and Chiba, 1949; and Kok, 1956). A similar difference between the effect of 'short' and 'long' flashes

was observed using isolated chloroplasts by Gilmour, Lumry, Spikes, and Eyring (1953). The flash bulb B_{60} is a 'long' flash and the electronic flash E_4 is approaching a 'short' flash. Table II compares the yield obtained in our experiments using single flashes with those observed by others with groups of flashes. The yields observed in our experiments, when there has been pre-excitation by diffuse illumination or a preliminary flash, are similar to those obtained by previous workers who used groups of flashes. This is a result of some interest since using only a single flash as in our experiments should minimize limitations on yield due to supply of carbon dioxide.

The introduction of a light activated catalyst of the type proposed above introduces a new constant into the formulation between average yield per flash and frequency of flashing for a group of flashes. The yield from any individual flash in a group will be determined by at least two terms: (*a*) the amount of free intermediate for activation which will be smaller the shorter the interval between this flash and the preceding flash, and (*b*) the amount of catalyst in the active form which will pass through a maximum during the interval following the preceding flash.

In the data for groups of 'long' flashes given in Table II it happens that the

TABLE II

Comparison of yield for a single flash with the average yield from a group of flashes

Single flashes

Type of flash	*Flash duration*	*Conditions*	*Flash yield moles chlorophyll/mole* O_2
E_4	100 μs.	Dark	∞
		Diffuse illumination	2,000
B_{60}	35 ms.	Dark	830
		Diffuse illumination	490
E_4 B_{60} (optimal spacing)	35 ms.	Diffuse illumination	350

Groups of flashes

Flash duration	*Dark interval*	*Authors*	*Flash yield*
10 μs.	10 ms.	Emerson and Arnold	2,000
8 ms.	400 ms.	Tamiya and Chiba	700
35 ms.	600 ms.	Briggs	250
35 ms.	400 ms.	Kok	435

dark times used were between 0·4 and 0·7 sec.—an interval such that the flash yields will be near maximal with respect to factor (*b*). The analysis of the mutual influence of these two factors in determining flash yield for 'long' flashes as a function of dark intervals is now being undertaken.

Another factor requiring further investigation is the relation between the intensity and duration of a first flash on the yield from a second. It would appear from our present data that the brief electronic flash must be repeated

several times (when there is no diffuse illumination) to obtain maximum yield per flash. On the other hand decrease in its intensity or lengthening the duration as in the flash E_{15} does not appear to affect greatly its enhancing effect on a following B_{60} flash.

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Observations on the Composition and Metabolism of the Nitrogen-Fixing Root Nodules of *Alnus*

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Received 15 January 1958

SUMMARY

Nodules of *Alnus glutinosa* (Alder) were exposed to excess ^{15}N either before or after detachment from the plant. The soluble nitrogen compounds were extracted from the nodules and the extracts fractionated by chromatography on an ion exchange resin. The amino-acid composition of the extracts was thus determined, and it was confirmed that citrulline is the predominant amino-acid present, being accompanied by smaller amounts of aspartic, glutamic, and γ-aminobutyric acids, arginine, and other constituents. The highest atom per cent. excess ^{15}N was always found in glutamic acid and the next highest in citrulline or aspartic acid. Ammonia contained a smaller proportion of ^{15}N than these compounds and arginine showed only very small enrichment. When the citrulline was degraded to ammonia and ornithine it was found that the ammonia liberated was richer in ^{15}N than even glutamic acid. The significance of these findings in relation to the fixation and further metabolism of nitrogen by the alder nodule is discussed.

INTRODUCTION

THE observations of Ferguson and Bond (1953), Quispel (1954), Bond (1955, 1957), and of Virtanen, Moisio, Allison, and Burris (1954) provide satisfactory proof of the occurrence of fixation of atmospheric nitrogen in the root nodules of alder at a rate adequate to support vigorous growth of alder plants in a rooting medium free of combined nitrogen. The fixation is presumably to be attributed to the specific soil micro-organism which inhabits the nodules; the identity of this organism is not finally established, though it is certainly quite different from the legume nodule organism.

The nitrogen metabolism of alder nodules thus acquires a special interest. Miettinen and Virtanen (1952) made a beginning in its investigation by carrying out semi-quantitative studies of the free amino-acids of the nodules, revealing the interesting fact that citrulline is the predominating acid. It accounted for about 0·25 per cent. of nodule dry weight in summer months, rising to nearly 2 per cent. in winter-time. Citrulline was also present in other parts of the alder plant, and in non-nodulated alders growing on combined nitrogen. Asparagine and glutamine were not detected, and the authors cited formed the opinion that in alder citrulline replaces amides in

storage and translocation. In further papers Miettinen and Virtanen (1953*a*, *b*) discussed the metabolism of citrulline in alder nodules and plants, but reached no very definite conclusions.

The present studies were designed to extend the above observations and also to attempt to gain some information about the mechanism of the fixation itself. It is well known that Wilson (1952) and Burris (1956) and their collaborators have gained new information on fixation mechanisms by the study of the distribution of fixed ^{15}N in cellular material after exposure to an atmosphere containing an excess of free ^{15}N. They have been led to conclude that ammonia is an intermediate in fixation and that it is the principal form in which the fixed nitrogen enters into organic combination. Similar isotopic studies are reported in the present paper with alder nodules.

METHODS

Culture of plants. The nodule material was provided by 5-month-old alder (*Alnus glutinosa*) plants grown in the greenhouse in water culture in Crone's solution pH 5–6 and free of combined nitrogen. Nodule development was secured by sowing the original seed in sand to which a suspension of crushed alder nodules had been added. The cultures were not of aseptic type, but abundant evidence has been obtained that the fixation is a nodular function as in legumes, and that contaminant organisms play no detectable part in the fixation (Ferguson and Bond, 1953; Quispel, 1954; Bond, 1957). At the time of use the plants ranged in height from 10 to 25 cm.

Exposure of nodules to excess ^{15}N. In some cases the fixation of ^{15}N was allowed to proceed while the nodules were still attached to the plant, in others after detachment.

For the exposure of attached nodules, plants were sealed with their root systems in individual wide-necked bottles partly filled with culture solution but with a 30 ml. gas space at the top, in which most of the nodules lay (Bond, 1955). Ten such containers were attached to a manifold and by suitable procedure the gas space in the bottles was charged at atmospheric pressure with a gas mixture comprising 20 per cent. oxygen, 10 per cent. nitrogen (with 36 atom per cent. ^{15}N), and 70 per cent. argon. The plants were left in the greenhouse under sunny conditions at a temperature of 21° to 27° C. for a period of 3 hours, after which the nodules were quickly picked off and placed in solid carbon dioxide to await extraction. A total of 40 plants was so treated over a period of 4 days, the total yield of nodules being 23 g. fresh weight.

Nodules to be exposed after detachment were picked from the plants as rapidly as possible and 1 g. quantities placed in Universal Containers of capacity 25 ml. Eight to ten such containers were attached to a manifold and charged at atmospheric pressure with a gas mixture similar to that indicated above, the closed containers with nodules being then incubated at 25° C. for 1·5 hours after which the nodules were placed in solid carbon dioxide. A total of 26 g. nodules was so exposed over a period of 2 days. All the above

exposures were made early in September 1955; in August 1956 and 1957 supplementary exposures of detached nodules were made, with a 30-minute period.

Extraction of nodules. Miettinen and Virtanen (1952) extracted the free amino-acids from alder nodules with 70 per cent. alcohol. These extracts contained a good deal of material which in air forms a dark-brown gum which interfered with later operations, and this material was separated from the amino-acids by means of Dowex 2 ion exchange resin. This procedure has two disadvantages. The resin would not be expected to retain, quantitatively, the basic amino-acids or the ammonia, and further, the Dowex 2 treatment in our experience causes the disappearance of some of the citrulline of the extract. Though the nature of the reaction involved has not been studied in detail the products include ornithine and some substance which travelled more rapidly than citrulline on a column of Zeokarb 225 and yielded citrulline and ornithine on being heated with acid.

Aqueous solvents do not appear to extract these gum-forming materials. In preliminary trials four extractants were compared, namely 0·1 N. and 3 N. HCl, 0·1 M. phosphate buffer pH 6·5, and water. Of these the first extracted the most nitrogen (namely about 20 per cent. of the total nodule nitrogen) and yielded a friable white powder on lyophilization. Since Miettinen and Virtanen (1952) have shown that amides are absent from alder nodules there appeared to be no objection to the use of an acid solvent, and 0·1 N. HCl was accordingly selected for use. Batches of nodule material of about 10 g. fresh weight were extracted with 9 times their weight of 0·1 N. HCl in an ice-cooled 'Nelco' blender for 5 minutes, after which the suspension was centrifuged and the residue extracted again with a half quantity of the HCl. The two extracts, which were slightly turbid, were combined and dried from the frozen state.

Fractionation of the extracts. The dried extract was taken up in 5 ml. of citrate buffer (pH 3·1) and the pH of the suspension adjusted to between 2 and 3 by addition of alkali. The suspension was then applied directly to a column (150 × 1 cm.) of Zeokarb 225 with nominal degree of cross-linking of 5 per cent., prepared and developed as described by Moore and Stein (1954). The effluent was collected in fractions of between 1 and 2 ml. volume. The absorption of ultra-violet light was measured at a wave-length of 260 mμ for each fraction, and aliquots of 0·1 to 0·2 ml. were analysed for amino-acids and related substances by the method of Yemm and Cocking (1955).

In analysing the nodule extracts, the position of a peak on the effluent curve was of assistance in the identification of the substance responsible, but since this type of extract had previously been studied only by means of paper chromatography (Miettinen and Virtanen, 1952) care was taken to obtain further evidence of identity of the fractions reacting with ninhydrin, by submitting them to paper chromatography before and after heating with 6 N. HCl at 115° C. for 15 hours.

Preparation of samples for isotopic analysis. The contents of tubes containing a particular amino-acid were mixed and the acid degraded to ammonia by

micro-Kjeldahl procedure (Hiller, Plazin, and van Slyke, 1948). Control studies showed that a 6-hour digestion was sufficient to avoid interference with the isotopic analysis (Rittenberg, 1946). The high concentration of buffer salt associated with the basic amino-acids emerging late from the column was removed prior to digestion by adsorbing the amino-acid on a short column (4×1 cm.) of Zeokarb 225 in the ammonium form. The column was then washed with dilute acetic acid and the amino-acid eluted with 0·05 N. ammonia, the latter being then removed by the addition of a little alkali and repeated evaporation in a desiccator. Effluent fractions containing ammonia were made weakly alkaline and the ammonia aspirated in a stream of nitrogen (ammonia-free) into boric acid.

The minimum amount of nitrogen required for a mass spectrometric assay was 0·4 mg., which exceeded the nitrogen content of many of the fractions obtained from 10 g. of nodules. Where at least 0·1 mg. nitrogen was available, ordinary ammonium sulphate was added where necessary to make up the nitrogen to 0·4 mg., a suitable correction being made to the mass spectrometric figure returned.

The authors are greatly indebted to Dr. A. S. McFarlane and Mr. G. Dickinson, National Institute for Medical Research, Mill Hill, and to Dr. R. I. Reed, Chemistry Department, University of Glasgow, for the ^{15}N assays. It should be noted that a difference of 0·010 atom per cent. in mass spectrometric values is easily significant.

Degradation of citrulline. This operation, the reason for which is explained later, was achieved by a modification of the method of Hirs and Rittenberg (1950). After removal of citrate from the citrulline fractions by addition of baryta followed by centrifuging, further baryta was added to the supernatant to give a concentration of 1·2 N and the solution refluxed for 20 hours, the ammonia thus formed being then carried over by a stream of ammonia-free nitrogen into 2 per cent. boric acid. The ornithine remaining in the reaction tube was subjected to the Kjeldahl process after the removal of barium as sulphate.

RESULTS

Composition of the extracts. Of the nodule material exposed in 1955 two 10 g. batches of attached nodules were separately extracted, and the same procedure followed with the detached nodules. Some bulking of nodules exposed on different days was necessary. The nodules exposed in 1956 were also subject to detailed analysis.

The result of the analysis of the extract from one particular batch (designated No. 1) of detached nodules is shown graphically in Fig. 1. The curve showing the degree of absorption of ultra-violet light suggests that besides simpler compounds the extract contained a complex mixture of nucleotides and related compounds. For the most part these were present in amounts insufficient for isotopic analysis and little effort was expended on their identification. Examination of absorption spectra between 200 and 300 mμ indicated

that most of the peaks represented mixtures but that three were more homogeneous and contained uridylic acid (peak A), cytidylic acid (peak B), and guanylic acid (peak C) or derivatives of these.

Fig. 1 also shows the distribution of substances giving colour with ninhydrin. In addition to free amino-acids the extract was found to contain a number of peptides. Those present in largest amount were concentrated in the earlier fractions astride the aspartic acid peak, and the shape of the curve suggests that there were at least five different substances present. There was insufficient material for characterization of these, and the pattern appeared to be rather different in other extracts, but paper chromatography of acid hydrolysates of these fractions indicated that the peptides had a relatively simple composition. All on hydrolysis yielded aspartic and glutamic acids, alanine, serine, and glycine, and some yielded also threonine and a basic amino-acid, either ornithine or lysine. Other peptides were found in very small amounts in later fractions, and one of these, appearing as a spike in the cystine peak in Fig. 1, was of interest as it gave on hydrolysis glutamic acid, ornithine, citrulline, hydroxyproline, and an unknown spot near the valine position on a two-dimensional chromatogram. Hitherto citrulline has not been encountered in combined form in plant tissues.

A certain failure of resolution was caused by the high concentration of citrulline in the extract, since the citrulline peak overlaps the positions occupied by the glycine and alanine, but this was not of much consequence since only traces of those acids were present. The separation of free ammonia caused some difficulty. In a test run with a known mixture, ammonia occupied the position given by Moore and Stein (1954), but with nodule extracts the position of the ammonia peak was found to be rather variable. In the first extract it overlapped the ornithine peak and in subsequent ones the γ-aminobutyric acid peak. As ammonia is not detected by paper chromatography the true position was not at first appreciated, and it was thought that there was too little ammonia present for detection. The overlap was discovered after the treatment of two batches of nodules had proceeded too far for the ammonia to be recovered. In later extractions the ammonia was separated from γ-aminobutyric acid by aspiration from alkaline solution, or, as in nodule batch No. 6 (see later), by making the extract slightly alkaline with calcium hydroxide before it was placed on the column, and aspirating the ammonia into boric acid.

The composition of the five extracts analysed in detail is shown in Table I. They all show a pattern similar to that already illustrated by Fig. 1. A large proportion of the nitrogen recovered (the latter being of the order of 12 per cent. of the total nitrogen in the extract) is present as citrulline, glutamic acid, γ-aminobutyric acid, and arginine. Smaller amounts of various other amino-acids and of ammonia are present. There is, however, a notable variation between extracts from different batches of nodules in the absolute amounts of the various amino-acids and in the total amino-acid content. Since the extracts were all prepared in exactly the same manner it seems that these

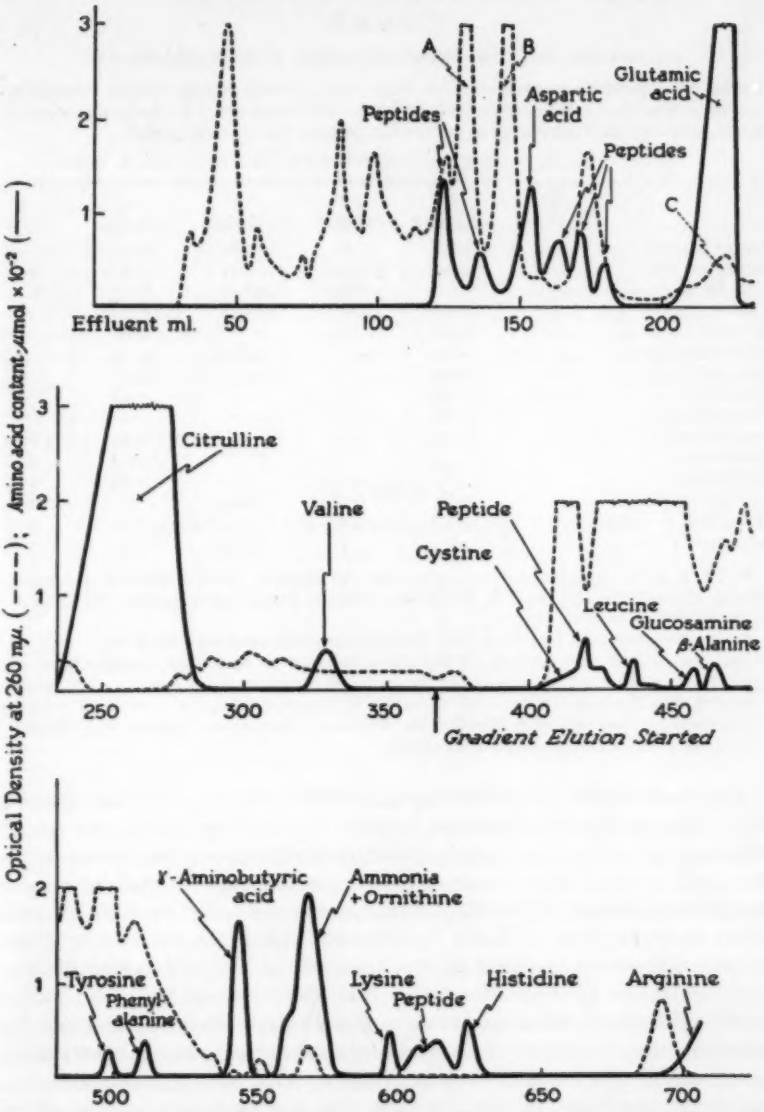

FIG. 1. Elution curves obtained in the chromatographic analysis of an extract from alder nodules (Batch 1).

variations must be due to physiological differences in the plants used, or to marked fluctuations in amino-acid content from day to day under the influence of climatic factors.

TABLE I

Amino acid composition of extracts of alder nodules

Results are expressed in terms of mg. N per 100 g. fresh weight of nodule material. (−) indicates that there was insufficient for confirmation. (+) indicates that the amino-acid was shown to be present but the amount was not estimated.

	Number and history of nodule batch				
	1 (D90) •	2 (D90)	3 (A180)	4 (A180)	5 (D30)
Aspartic acid†	0·84	+	0·82	0·49	+
Glutamic acid	4·65	1·54	1·71	1·32	1·90
Citrulline	53·0	5·60	17·20	9·05	6·64
Valine	0·27	—	0·21	0·05	—
Cystine	0·70	—	—	0·06	—
Isoleucine	—	—	0·09	0·13	—
Leucine	0·10	—	0·08	0·17	—
Tyrosine	0·27	0·47	—	0·18	—
Phenylalanine	0·18		—	—	—
γ-aminobutyric acid	1·15	0·31	4·82	1·34	1·19
Ammonia	2·39	2·17		3·04	0·80
Ornithine		0·67	0·70	0·06	0·20
Lysine	0·70	0·31	0·53	—	0·24
Histidine	1·70	0·63	—	—	0·25
Arginine	+	+	3·52	3·52	1·18

• 'D' and 'A' indicate respectively that the nodules were detached or attached during exposure to excess ^{15}N, while the number following indicates the period in minutes of exposure to the ^{15}N.

† The aspartic acid fractions were slightly contaminated with peptides.

Note. Serine was also present in the extracts but was seriously contaminated with peptides, so that no entry is made for it. In the extract from batch 1 only, glucosamine and β-alanine were also present, in concentrations of 0·07 and 0·15 mg. nitrogen per 100 gm. nodule material. Traces of glycine and alanine were found in batches 1 and 4 but were not estimated.

Mass spectrometric data. The original intention was to pool similar fractions from different batches of nodules in order to provide sufficient nitrogen for ^{15}N assay of as many fractions as possible, but in view of the variability just discussed it was thought better to have separate assays for each batch, even though this reduced the number of fractions available for assay. The results of the assays are given in Table II. Data available in the case of batch No. 4 indicate that some 60 per cent. of the excess ^{15}N present in the original extract is accounted for by the fractions shown in Table II together with additions calculated from other batches for the γ-aminobutyric acid and ammonia. It is presumed that there was similar recovery in the other batches. It should be noted that though fractions containing amino-acids sometimes also contained substances absorbing ultra-violet light, this contamination was insufficient to affect isotopic analysis. For instance, if the substance overlapping the glutamic peak (Fig. 1) is assumed to be guanylic acid, the ratio of guanylic acid-nitrogen to glutamic acid-nitrogen is only 1:400.

In general the enrichment shown by the amino-acids is greater in the nodules which were in the detached state during exposure to the ^{15}N. It

TABLE II

Distribution of ^{15}N in the constituents of the nodule extracts

Results are given as atom per cent. excess over the normal value (0·375 atom per cent.). Batch number conventions as in Table I. Aspartic acid is slightly contaminated with peptide.

	Number and history of nodule batch					
	1 (D90)	2 (D90)	3 (A180)	4 (A180)	5 (D30)	6 (D30)
Aspartic acid	0·910	—	0·208	0·210	—	—
Glutamic acid	1·199	0·956	0·451	0·427	0·250	0·156
Citrulline	0·590	0·873	0·241	0·342	0·179	—
γ-aminobutyric acid	0·660	0·348	0·176	—	—	—
Ammonia	—	—	—	—	0·187	0·040
Arginine	—	—	0·014	0·012	0·012	—
Peptides *a*	0·291	—	0·090	0·078	—	—
Peptides *b*	—	—	0·159	0·058	—	—
Ultra-violet absorbing fractions i	0·225	—	0·088	—	—	—
Ultra-violet absorbing fractions ii	0·588	—	0·055	—	—	—

TABLE III

Distribution of ^{15}N in citrulline isolated from nodule extracts as atom per cent. excess

	Number and history of nodule batch	
	4 (A180)	5 (D30)
Citrulline, total found	0·342	0·179
Citrulline, total calc.	0·324	0·181
Carbamino-N	0·578	0·390
Ornithine-N	0·197	0·076

would be difficult to forecast what such a comparison would show. The rate of fixation is undoubtedly much reduced by detachment (Bond, 1957), but on the other hand it is obvious that all the nitrogen that is fixed is retained in the nodules, whereas this is not true of attached nodules. It has also to be remembered that the ingress of organic compounds to accept the products of fixation cannot occur in detached nodules.

The distribution of ^{15}N among the fractions analysed is, however, similar, whether the nodules were attached or detached during exposure. The glutamic acid shows the highest enrichment in each batch of nodules and thus exceeds the citrulline, though this also shows substantial labelling. As already mentioned, the citrulline peak overlapped very small peaks due to glycine and alanine, but the possibility that the presence of these substances would affect the isotopic assay of the citrulline fraction was excluded by dividing the citrulline-containing tubes into groups prior to assay. The groups were assayed separately but showed no significant difference in ^{15}N content. Other compounds with a relatively high labelling are aspartic acid and γ-amino-butyric acid, both of which are presumably in close metabolic relationship

with glutamic acid. The ultra-violet-light-absorbing fractions and the peptide fractions which were assayed showed lower labelling than the free amino-acids.

The free ammonia fraction from batch No. 4 was unfortunately lost, but the data secured for batches Nos. 5 and 6 show that the enrichment of the ammonia was considerably less than that of the glutamic acid.

As citrulline proved to be markedly enriched it was thought desirable to determine how the isotope was distributed among the nitrogen atoms of the molecule. Portions of the citrulline fractions from two batches of nodules were therefore degraded, in the manner described earlier, to ornithine and ammonia. Mass spectrometric assay (Table III) showed that the ammonia-nitrogen, derived from the carbamino group of the citrulline, was much more strongly labelled than the ornithine-nitrogens. Moreover, comparison with Table II reveals that the labelling of this carbamino-nitrogen exceeded that of glutamic acid-nitrogen.

DISCUSSION

As in legume nodules, the actual site of the fixation in the alder nodule is conjectural, but the general circumstances suggest that the fixation is achieved inside or on the surface of the endophyte. The latter, though it has not been isolated, has been considered by many but not all cytological observers to be actinomycetal in nature. It is certain that the great bulk of the fixed nitrogen is quickly acquired by the host cytoplasm and conveyed out of the nodule to the rest of the plant (Bond, 1956), but a substantial part of the total nitrogen present in the nodule at a given time must be in the endophyte, so that the fractions examined in the present work are liable to be derived from two sources—the endophyte and the protoplasm of the nodule cells. It seems probable, however, that the bulk of the soluble nitrogen will be derived from the latter source, since it is there that the greater part of the fixed nitrogen is further metabolized and prepared for transport.

The data now reported show, in agreement with Miettinen and Virtanen (1952), that citrulline is quantitatively the most important free amino-acid in alder nodules. The actual amount present was very variable and ranged from 6 to 53 mg. of citrulline-nitrogen per 100 g. fresh nodules in samples collected from apparently fairly uniform plants over the space of a few days in September. Values obtained by the Finnish authors and expressed in the same terms were 120, 12, and 18 mg. for nodules collected in January, May, and June respectively.

Appreciable quantities of glutamic, aspartic, γ-aminobutyric acids, and ornithine and arginine were also detected in the present material. Citrulline and the last two amino-acids are usually considered a closely related group. Arginine is an important constituent of proteins, but citrulline and ornithine have until recently been best known as intermediates in urea synthesis in mammalian liver. It has been suggested that the ornithine cycle is a special adaptation of a mechanism primarily of importance as a means of arginine synthesis (Ratner, 1956).

Meanwhile instances of the occurrence of citrulline in plants are mounting in number. Since its detection in alder, citrulline has been found in the xylem sap of birch, hazel, walnut, avocado, and other woody plants (Reuter and Wolffgang, 1955; Bollard, 1956, 1957), while Linko, Holm-Hansen, Bassham, and Calvin (1957) have shown it to be present in blue-green algae. Its occurrence in this wide range of plants shows that citrulline has no specific connexion with nitrogen fixation. It appears to serve for the translocation and storage of nitrogen, but its origin and fate and whether it is concerned in a cyclic process as in animal tissues have remained uncertain (Steward and Pollard, 1957). The data in Table II show that citrulline in the alder nodule is metabolically active, and in view of its high concentration in the nodule this substance contains a considerable proportion of the fixed ^{15}N recovered. The data also show that though arginine may be formed from citrulline in the alder nodule, the rate of this reaction is very small compared with that of the formation of citrulline. It would appear therefore that the main importance of citrulline in the nodule lies elsewhere than in connexion with arginine synthesis, and our observations are consistent with its being concerned with translocation and storage of nitrogen.

The distribution of ^{15}N in the citrulline molecule is of interest. The carbamino-nitrogen of the citrulline was more heavily labelled with ^{15}N than the ornithine-nitrogen of the citrulline, irrespective of whether or not the nodules were attached to the plant during exposure to excess ^{15}N. This suggests that there existed a reservoir of precursors for the ornithine portion of the molecule, whereas the carbamino-nitrogen is derived more directly from the fixation process. In animals and bacteria it is held that the formation of citrulline proceeds by the reaction of ammonia with carbon dioxide to form carbamic acid, which after conversion to carbamyl phosphate reacts with ornithine to give citrulline (Jones, Spector, and Lipmann, 1955). In plant material Linko *et al.* (1957) observed that when *Nostoc* was exposed to $^{14}CO_2$ for a short time, the ^{14}C subsequently detectable in the citrulline molecule was restricted almost entirely to the carbamino-carbon. The differential labelling of the citrulline nitrogen atoms detected in the present study offers a striking parallel to the finding with *Nostoc*. Both observations are consistent with the idea that citrulline is formed in plants by a mechanism similar to that proposed by Jones *et al.* (see above).

For the citrulline to have such an origin the presence in the nodules of highly labelled ammonia from the fixation process would be necessary. The same argument may be derived from the relatively high labelling of glutamic acid which may well be produced by amination of α-ketoglutaric acid. The free ammonia fractions did not, however, show the degree of labelling required by the hypothesis that ammonia production precedes the formation of these amino-acids. The same difficulty was encountered in several of the experiments of Wilson and Burris and their collaborators (see Introduction) with other nitrogen-fixing material. In view of other evidence indicative of the formation of ammonia as an intermediate between fixation of nitrogen and

incorporation into organic molecules, they concluded that the glutamic acid (the fraction which showed the greatest enrichment in all their material) arose by amination of α-ketoglutaric acid by a highly enriched ammonia fraction which itself became diluted with other ammonia during extraction. Our findings could be explained in the same way, but it should be noted that this demands that the tissues in close relation to the fixation process are richer in amino-acids relative to ammonia than the tissues which contribute the inert ammonia.

Thus the data obtained for alder nodules are consistent with but do not prove the theory that ammonia is an intermediate in fixation and is the form in which the nitrogen enters into organic combination. Unequivocal proof that ammonia has this role in nitrogen-fixing organisms may only be obtained when fixation can be brought about in a cell-free extract.

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The Translocation of Sugar in Sieve Tubes

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SUMMARY

A theory is put forward to account for the translocation of sugars in terms of electrokinetic forces developed in the cytoplasm of the sieve plates. A major role is suggested for potassium as responsible both for the movement of solution and for maintaining the necessary potentials across the sieve plates. The mechanism for the latter invokes the active uptake of potassium (or some other ion) by the companion cells. It is suggested that in the overall process of conduction there are features which can be described as involving the elements of inertia and positive energy feedback, and that a possible result of these would be longitudinal oscillations in the phloem columns which might assume importance as the seat of rhythms and as a background for photoperiodic effects, and which might be adjusted to an imposed period by callose constrictions. The mechanism put forward would seem to be physically adequate even for very lengthy phloem columns, and it would seem possible for it to accommodate most, if not all, of the major experimental facts. It implies the active transport of water, as well as of sugars and other chemical species.

THE problem of sugar transport in the higher plants is one of the classical problems of plant physiology, yet in spite of a large amount of positive knowledge about it which has been accumulated over a considerable number of years the mechanism involved is still largely a mystery. It is not my purpose in this paper to review the subject, as a number of excellent recent reviews exist already (Arisz, 1952; Crafts, 1951; Esau, Currier, and Cheadle, 1957), but a short recital of ideas currently held and of the objections to them may help to set the matter in perspective.

The most favoured view is probably represented by the pressure-flow theory of Münch. According to this theory as usually understood there is a bulk flow of the sieve-tube contents down the columns under the influence of a gradient of hydrostatic or turgor pressure. This flow traverses, of course, the sieve plates. The pressure gradient falls continuously from the source—usually the leaf—to the sink, usually a young embryonic region; and it is established and maintained osmotically. At the extreme ends of the columns metabolic processes may be at work, as sugar is discharged into the head sieve tubes or withdrawn from the foot ones; but the overwhelming proportion of the distance covered, that is of the resistive path, is traversed under the influence of a simple pressure gradient. Transport is, therefore, thermodynamically passive rather than active.

In spite of very solid evidence in its support Münch's theory runs up against several severe difficulties. Electron microscope and other evidence would seem to indicate that the sieve plate pores are filled by fairly dense

cytoplasm. If this is stationary there, the pressures required to maintain the observed rates of flow through stems and roots of any appreciable length would be vastly too great to be conceivable in the living plant; whereas if the cytoplasm moves with the stream very large forces would still be required, and in addition there would be the problem of the fate of the living matter. Further, the pressure gradients experimentally observed do not always seem to be in the direction required. Nor does the theory do justice to the great body of evidence which indicates that the phloem function is essentially a *living* one. Experiments on oxygen requirement, and on the effects of narcotics, poisons, temperature variations, and heat treatment have established this beyond any reasonable doubt. It is true that Münch's theory requires a barrier around the conducting tissues relatively impermeable to sugars, but this feature hardly constitutes a link with the physiological requirements adequate to the facts of the case. Finally, it is not easy to accommodate the evidence for simultaneous two-directional movement in the phloem; nor for transport of different materials at different rates, unless, as will be suggested later, the cytoplasm is stationary in the pores of the sieve plates. But this latter supposition involves the theory as mentioned above in quite unsurmountable difficulties of its own.

The second current theory of transport invokes cytoplasmic streaming, combined perhaps with simple diffusion across the sieve plates. This proposal is more definitely physiological, and meets some of the objections to the former hypothesis. But it fails in two major respects. Streaming has never been observed in mature sieve tubes; and even if it had, the necessity for flow and return paths would so reduce the cross-section for transport that streaming velocities would be required far in excess of those normally associated with cyclosis.

The Münch hypothesis and the streaming hypothesis, or variations of them, are really the only two current suggestions which are at all feasible. The supposition of some sort of 'activated diffusion' or of movement along interfaces, if these rather vague suggestions have been rightly understood, would involve massive transport of sugar through a roughly stationary matrix, and the overall energy requirements for this would be fabulous. Unless these mechanisms are limited to a very minor role, therefore, they can be definitely ruled out.

In passing on now to my own speculations I shall take a number of claims as established beyond reasonable doubt: that massive sugar transport takes place predominantly in the sieve tubes; that it is, unlike movement in the xylem, a definitely living activity; and that it is a key process often determining by its intensity and direction the movement of such diverse elements as viruses and synthetic hormones.

In the light of these facts it seems to me that an essential feature of any acceptable theory of sugar transport must be that it involves bulk flow of the contents of the sieve tubes at a rate substantially the same for all the major components, and that this flow must include passage across the sieve plate.

I want to suggest, however, that this flow is actuated not passively as Münch supposed, but actively, and by a mechanism located at the sieve plates. A possible form which this mechanism might take will be described later; but there are one or two general points that arise immediately. The first is that the general pattern of hydrostatic pressure change along the sieve tube columns

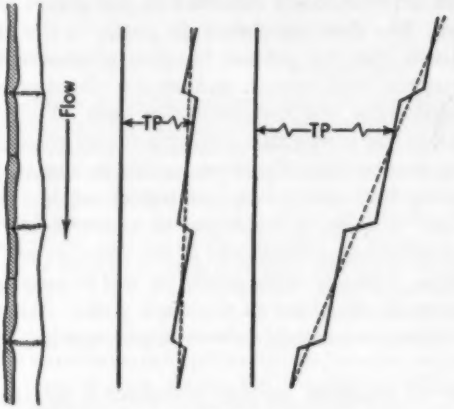

FIG. 1.

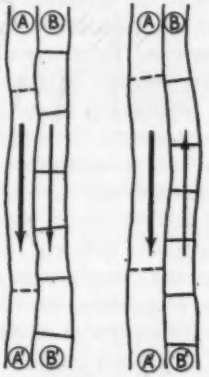

FIG. 2.

FIG. 1. Turgor pressure variation (TP) along the tubes is shown (right) for the pressure-flow theory and (centre) for the present theory. Overall gradients are shown dotted.
FIG. 2. Primary flow (heavy arrows) and induced flow (light arrows) in a passive system (left) and active (right).

will follow a saw-tooth profile (Fig. 1) instead of a continuously falling one as in the pressure-flow hypothesis. Between the sieve plates the pressure will fall in the direction of movement because of the viscous resistance, the flow here being in fact thermodynamically passive. But across the sieve plates the pressure will rise on account of the active forces, and the rise may more, or less, than offset the previous fall. Thus the overall gradient in the columns will not necessarily be a positive one, though there are grounds for thinking that it usually will be so.

A theory based on these general lines obviously meets at once two major objections to the Münch theory; it is highly physiological, and it calls for no excessive pressures. But it has other immediate advantages. There is an inherent difference between a mechanism predominantly active, and one predominantly passive. Longitudinal distances are, in the present context, very much greater than transverse ones, and it is therefore reasonable to assume that the rate of transverse equilibration, with respect for instance to such a variable as suction pressure, will be considerably greater than that of longitudinal equilibration. Consider two adjacent longitudinal rows of cells—it does not matter whether they are living or dead, specialized or unspecialized (Fig. 2). Suppose that there is a passive movement of water down one row AA'. By definition of passive flow, the suction pressure at A' must be higher

than that at *A*. From what has just been said it will follow that the suction pressure at *B′* will be higher than that at *B*, *B* and *B′* being at the same levels respectively as *A* and *A′*. At least this will be so unless the resistance to lateral movement of water is relatively very high. In ordinary cases, therefore, the streaming from *A* to *A′* will be accompanied by a streaming from *B* to *B′*. This parallel movement in the same direction, even if it is masked by other forces, represents a tendency which is necessarily present wherever a passive mechanism is at work; the ascent of sap in the xylem, for instance, must be accompanied by an upward movement in the cambium, unless indeed other movements there mask it. Even so, the *tendency* will be present.

In the case of an active mechanism the case is, however, different. The suction pressure at *A* may now be either greater or less than that at *A′*; if it is greater there will be no question but that the movement is an active one, and in fact no loss of generality will be involved if for simplicity we choose this case for consideration. The suction pressure at *B* will then be greater than that at *B′*, and in the absence of the active mechanism in the row *BB′* there will result a passive flow in the reverse direction, i.e. from *B′* to *B*. Thus while a passive flow is always accompanied by a tendency to a parallel flow in the *same* direction in adjacent channels, an active flow, or rather component of flow, is invariably accompanied by a tendency to a *reverse* flow. This flow may be merely local; but if the overall gradient (cf. Fig. 1) is negative it may take place over long distances.

The relevance of these considerations to the problem of translocation is not difficult to see. In any phloem bundle there are elements of different sorts and of different sizes: sieve tubes, companion cells, and parenchyma. Even within the single category of sieve tube it is not necessary to hold that all channels have the same activity. Provided only that movement is under the control of active forces and that some lines are relatively less active than others, it will follow that some channels may experience a reverse flow. Especially will this be so if, as will be suggested later, the condition of zero flow in the tubes is, on account of the presence of an active mechanism, an unstable one, so that a flow, once started, tends to grow stronger; and once reversed tends to stay so. It is here that an explanation may be envisaged for the phenomenon of independent movement or two-directional flow which seems fairly well established. What the evidence apparently shows is that solutes carried in relatively small amount, such as ions, dyes, and perhaps even organic nitrogenous materials, can travel in the phloem in a direction opposed to that of carbohydrate transport. On the present suggestion this would present no insuperable difficulty, unless indeed it could be shown that the reverse movement occurred in the same sieve tubes as the direct one; but this has apparently never been done, even with dyes.

What sort of active mechanism can be advanced as responsible for the flow of bast sap? I should like to suggest the possibility of an electro-osmotic one.

It is not generally realized how very strong electro-osmotic forces can be. An illustration frequently used as a class experiment (after Loeb, 1919) can

be used to demonstrate this point. An ordinary thimble of collodion, cast in a test-tube, is taken and fixed to a capillary glass tube with a rubber bung. It is then filled with distilled water and immersed in various solutions, the rate of movement of the meniscus in the capillary being taken as a measure of the 'osmotic power' of the solution.' When this is done and the rates recorded using sucrose and various salts, and allowance made for the dissociation of the latter, the sort of curves shown (Fig. 3) are obtained. It will be seen that the rates of movement induced by quite low concentrations of sodium citrate, for

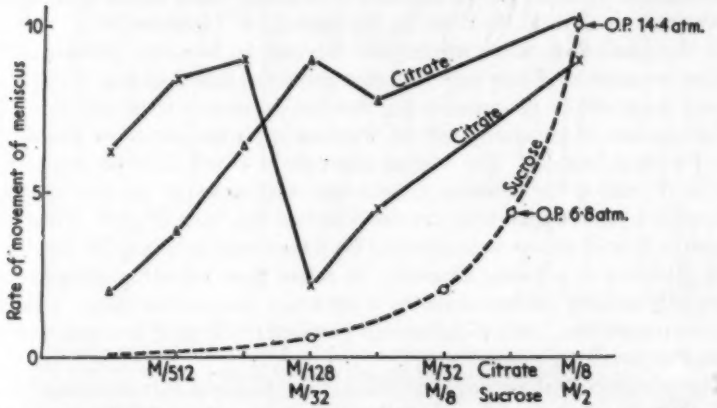

FIG. 3. Osmosis of water from a collodion thimble into citrate and sucrose solutions.

example, are vastly greater than can be accounted for by their osmotic effect; in fact M/512 citrate may have an effect equivalent to that of M/2 sucrose, that is of about 14 atmospheres. The direction of movement in the examples shown is positive; it reinforces normal osmosis. But treatment of the collodion membrane with a suitable solution, such as dilute gelatine, can quite easily reverse it, the membrane charge changing sign and so-called negative anomalous osmosis resulting.

The requirements for an effective electro-osmosis are briefly three: a charged membrane (and very few membranes in aqueous systems do in fact escape becoming charged); a membrane potential, that is a difference of potential between its two sides; and a porous structure in the membrane coarse enough to allow the passage of highly hydrated ions without being so coarse as to allow them to pass out of range of the electric fields of its fixed charges. Provided these requirements are met by the sieve plate, and provided energy is available for continuously maintaining the membrane potential (which of course tends to become discharged as a result of the electro-osmotic movement) there would seem to be no reason why an effective action should not be sustained, and sustained fairly economically from the thermodynamic point of view.

The suggestion is therefore that the complex, living sieve plate itself

constitutes such a membrane, the electro-osmotic forces being developed in the interstices between the fibrils of the dense cytoplasm which apparently fills the sieve pores. Before such an idea can be given credence, however, a number of aspects must be examined, and even then it must be recognized as frankly speculative.

In the first place, is such an arrangement capable of developing sufficient effort for translocation? From what has already been said it would appear that there is no reason, *a priori*, to doubt this. The electrical forces can, as we have seen, be very considerable, and so far as the passage of the sieve tube 'internodes' is concerned the path is relatively unresistive. The sieve plates are a different matter, but even here calculation shows that an equivalent effort of about an atmosphere would drive a 20 per cent. sucrose solution across a single sieve plate with pores no larger than 0·02 μ diameter at speeds of the required order (that is, corresponding to a mean velocity down the entire columns of 30–100 cm./hour). Such a requirement of course could not conceivably be accommodated by the Münch hypothesis; but it presents no very serious problem to electrokinetic forces developed in the manner suggested.[1] In this connexion lengthy stem and root systems suffer no disadvantage as compared with short ones. The total available effort, unlike the state of affairs in the pressure-flow hypothesis or for that matter the cohesion theory of xylem transport, increases proportionately to the path length and the problem of transport is no more serious for a tall plant than for a short one. There is a further point of interest. The motive forces are developed in such a way that although so large they exert no tendency to drive the cytoplasm occupying the pores in the direction of flow. As a matter of fact there would probably be merely a slight resultant pressure exerted on the living matter of the sieve plate in the reverse direction, consequent simply on the necessity for a turgor gradient to propel the solution along the body of the sieve tube. So far as the actual passage of the sieve plate is concerned the system of forces involved could be compared with those called into play when a man, with one hand pressed against a mesh in a wire fence drags through that mesh a piece of rag. He might have to exert himself very appreciably, but the fence as a whole would, if he did the job properly, experience no unbalanced effort. This conclusion again distinguishes the suggested mechanism rather sharply from that of Münch, where of course the cytoplasm on the sieve plates has to sustain itself against the full force of the mass flow, much like a water plant anchored to the bed of a swift torrent; unless, indeed, as has been suggested, it is itself translocated and utilized elsewhere.

In the second place, can the idea be seriously entertained that arrangements exist in the phloem for sustaining a continuous electrical potential across the sieve plate? I think it will be agreed again that this point does not, on the

[1] In the simple system of Fig. 3 the diffusion of ions both sets up the membrane potential and effects the transfer of water. In the scheme suggested below the membrane potential is independently and actively established, resulting in a system of both greater power and greater flexibility.

surface, present much difficulty. Highly active living cells are an invariable and intimate accompaniment of the sieve tubes. The companion cells of the angiosperms have been shown to possess high enzymatic activity, and the phloem as a whole a high rate of oxygen consumption. It would seem entirely possible that these chafacteristics of the companion cells might be associated with a high activity in the active uptake of ions from the sieve tubes. If that were so, one need only postulate that the activity was different, perhaps only slightly different, on the two sides of the sieve plate for the electrical requirements to be met. I should like to pursue this speculation to the point of suggesting the following scheme, local circulation of potassium both sustaining the membrane potential and transmitting the motive effort to the sieve tube contents (see Figs. 4 and 5). Potassium would seem to be a suitable ion for the mechanism. It is present in large enough amounts, is highly mobile, and is known to be subject to active uptake. Further, for our comfort, it is a major essential element for which no adequate role has yet been confirmed; moreover, certain observations on the effect of potash starvation can be interpreted sympathetically to the present suggestion, though one has to be very guarded here. The electro-osmotic flow thus established would as indicated have to be accompanied by a continuous reverse flow of potassium ions, the return path lying in the cytoplasm of the companion cells, and the question arises, are these large enough to sustain this? It is difficult to say how many molecules of sap would be moved per potassium ion; it would in any case probably vary with the resistance encountered. It is a question of how many would be electrostatically held by the field of the ion, and of how many more would be dragged along frictionally by its motion. On the average it would seem that to assume a ratio of 100:1 or perhaps even more would not be unreasonable. The cross-section of the companion cells is of course much smaller than that of the sieve tubes, but a current of the order indicated would not seem impossible. The actual velocity of movement of the ions within them need not of course be very large; if the companion cells were rich in potassium a very small velocity would suffice to ensure the necessary current.

According to these suggestions when conduction is in progress the sieve plates are electrically polarized, and needless to say all plates belonging to the same file would be expected to be polarized in the same direction. What could lead to this latter state of affairs? It might of course be inherent in the overall structure of the plant, or it might be due to the unidirectional movement of some hormone; but in view of the experimental fact that it is fairly easy to reverse the carbohydrate stream by manipulating sources and sinks—for example, by cutting off leaves or young fruits—it is tempting to assume that it is the developing sugar gradient that in fact acts as 'signal' and determines both the magnitude and direction of the transport. It can be suggested that transport might then be established in some such way as the following (Fig. 4). Starting from a quiescent state, the discharge of sugar (1) into the head sieve tubes from a source would lead to an immediate rise of suction pressure in the tubes. Following this water would be withdrawn from the upper ends of the

columns of companion cells (2), and this would lead to a vertical surge (3) of the contents of the companion cells. In this movement the freely mobile potassium ions would doubtless take part. As a result the actively maintained potassium balance between companion cells and sieve tubes would be locally disturbed, in one direction at the upper half and in the other at the lower. Fluxes of potassium ions (4) would thus become initiated, and the sieve plate would become electrically polarized. Other effects of course might also be operative —a partial inhibition of the ion absorption mechanism by the considerable rise in sugar concentration, or a rise in viscosity or in permeability to ions due to the same cause. These might be important in later stages. The further suggestion can now be made that the quiescent state of the sieve tubes is an unstable one, and that the stable states are fully polarized. This could result from a sort of positive feed-back. Once electro-osmotic action has begun it is only necessary to assume that some molecular species present is preferentially conveyed across the sieve plate (owing perhaps to its closer association with the ionic prime movers or through differential filtration) and that this preferential movement establishes a concentration difference for this species which augments the divergence already existing between the intensities of the active ion uptakes on the two sides. A very simple possibility would be that electro-osmosis conveys water across the sieve plate faster than sugar. This would lead to an augmented and permanent differential in sugar concentration, which might have the desired effect on the active mechanism. A reason for the preference of the translocatory mechanism for sucrose may be sought here; its larger molecule may lag more significantly than the smaller hexoses. The importance of this possible effect can be readily seen. It would establish full polarization of the first sieve plate as a stable state; and even more important, it might rapidly effect a congruous polarization in succeeding sieve plates. They would only need to experience a slight osmotic unbalance for them to respond, and with each response the movement would gain force until translocation was in full swing, a state of affairs which can be illustrated by a diagram (Fig. 5). As a corollary to this scheme, it would be anticipated that when the source ceased to function an effect analogous to inertia would become apparent, transport continuing as before until the sugar in the sieve tubes had been redistributed longitudinally in such a way as to develop an overall reverse gradient steep enough to overcome the polarization of at least some of the sieve plates. The movement would then begin to draw to a close, until restarted by the beginning again of source activity. Sink activity, of course, would have a complementary importance in all this.

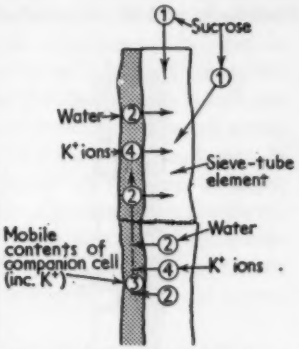

FIG. 4. Initiation of sieve-tube activity. (See text).

An interesting possibility arising from the suggestion that the polarized states of the sieve plate might be the stable ones and the neutral state the unstable one is that under certain circumstances flow in the phloem might be oscillatory in nature. The system suggested does, as mentioned, involve positive feed-back, and the element of 'inertia' is present; it would not be surprising

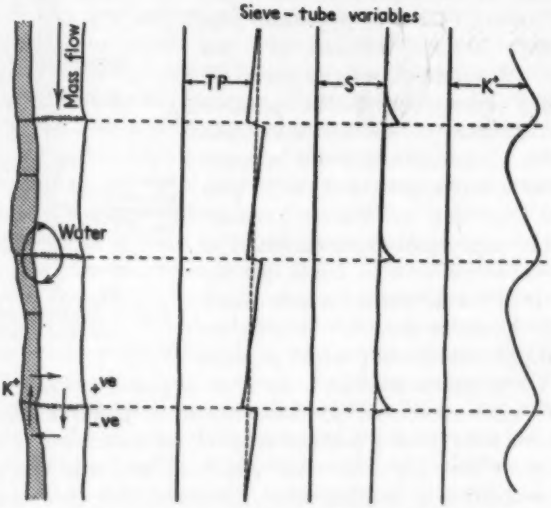

FIG. 5. Left, local circulations of water and potassium ions. Right, variations along columns of turgor pressure (TP), sugar concentration (S), and potassium ion concentration (K^+).

therefore if such a state of affairs did in fact occur. The period would depend, among other things, on the length of the reacting columns, and these might be influenced by the partial occlusion of occasional sieve plates with callose. To carry speculation a stage further, the system might by some such means as this be 'turned' to a 24-hour or similar cycle, setting the stage for the autonomous rhythms so often found in plants, and providing a background for photoperiodic behaviour. These speculations are perhaps at this stage a little wild; but they would not seem to be altogether outside of the range of possibility. Incidentally callose formation in winter in such plants as the vine would put a stop to movement, direct or oscillatory, and enable sugar concentrations to be fixed in different regions independently. Teleologically these considerations might seem to be a justification for callose.

It has already been hinted that the proposed mechanism, though involving mass transport, does not imply that every molecular species present in a sieve tube will move at the same rate. Rather, in some respects the whole process may be likened to chromatography or differential filtration. Viruses, for instance, might be expected to move less rapidly through the congested sieve pores than smaller molecules, and the same applies to ions—probably anions—

bearing the same charge as the structure. On the other hand cations might well move faster. Experiment seems to confirm some of these expectations.

In conclusion I should like to make some general remarks about the proposed mechanism, which might be said to constitute the 'electro-osmotic theory'. I think it meets all the major requirements demanded by the evidence. It is physically on the right scale; it is highly physiological; and it invokes, in what I hope is a credible fashion, well-known forces of adequate power for the task. It does not seem to involve any structural requirements which are not, in fact, matters of common knowledge; and conversely it assigns a role for most major features of the phloem which would seem to demand one. It possesses, in addition, features which are suggestive of answers to other problems: the seat of autonomous rhythms; the role of potassium in the plant; the function of callose; the preference for sucrose; and, through the implication of enzymes in active ion uptake, the possible significance of such trace elements as boron. Many of these suggestions, however, are by no means essential to the theory.

The free-energy supply is of course mediated through the system responsible for the active ion uptake, the ions being absorbed from a low concentration and released into a higher one. These energy relations clearly need looking into more carefully, by no means a simple task.

Should the theory in its essentials be able to establish itself in the light of further evidence it would mean that a step forward has been taken not only in the understanding of translocation but also in one or two other important matters; positive feed-back, so important a principle in animal physiology, would have put in an appearance in the plant world, and the debate as to whether or not active transport of water occurs in plants would have been carried a stage further. But that is perhaps looking rather far ahead.

Note. Since this paper was written my attention has been drawn to an interesting article by D. S. Fensom in the *Canadian Journal of Botany* (**35**, 573, 1957). Fensom discusses briefly the possibility that electrokinetic forces may play a part in both xylem and phloem transport. He concludes that they may be important in the latter, but limits himself to the suggestion that they operate in the sieve-plate pores, not in the far finer channels of the cytoplasmic plugs. As a result the forces developed, as well as the resistances overcome, are far smaller than in the present theory. The driving potential, he supposes, may be due to the unequal oxidation of sugars with the production of organic acids, whose hydrogen ions diffuse through the sieve plates. These interesting ideas have provoked me to think on the possible value—or otherwise—to the plant of having the sieve pores plugged with cytoplasm, as they would seem to be. On the one hand this factor obviously increases enormously the resistance to flow; on the other hand it very greatly enhances the mechanical effort and thermodynamic efficiency of the electrokinetic forces. Which of these two effects would predominate is hard to say without careful investigation; but one would certainly guess the latter. Further, the presence of the

cytoplasmic plugs not only introduces the element of possible metabolic control of the zeta-potential of the living sieve plates; it also raises the electrokinetic forces to a height which assures their ascendency over purely hydrostatic ones. The result is that the overall pattern of transport in the plant is secured more firmly in the control of physiological forces than it would otherwise be; local changes in turgor, such as the temporary wilting of leaves, are therefore less likely to cause disorganization. Over and above these effects is the very suggestive one that the much narrower pores through the cytoplasm can introduce an element of discrimination towards different molecular species. It is this factor which, it has been suggested, allows the possibility of positive feed-back with its important consequences. One of these not hitherto mentioned is that it makes it easier to conceive how a translocatory mechanism, at least superficially rather similar, could have arisen independently in such diverse groups as the brown algae and the higher plants. If positive feed-back operates as suggested any tissue possessing a *tendency* to conduct would find itself, ontogenetically speaking, positively encouraged to do so; and its ability to respond might have consequences for phylogeny.

Professor A. S. Crafts has raised verbally the valid objection to the suggestions of this paper that in some plants, at least, the companion cells (or their analogues in plants which do not possess them) are in contact with only a short portion of each sieve-tube element and do not 'straddle' the sieve plates as the requirements of local potassium circulation would seem to require. I can only suggest that such plants possess some more roundabout way of returning the potassium (or other ion) from one side of the sieve plate to the other. This suggestion might apply also to the brown algae.

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The Effects of Growth Regulators on the Geotropism of Leaf Sheath Basal Region in Wheat Plant

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SUMMARY

The present study was carried out to ascertain the influence of growth-regulating substances on the intercalary growth region of the wheat leaf sheath. Stem segments containing a node were used.

The growth-stimulating substances, IBA, NAA, and 2:4-D, and the growth-inhibiting substances, TIBA and DCA, were mutually antagonistic in their actions on the growth of this region. From the result of histological observation, it was indicated that the growth-stimulating substances promoted the cell elongation of this region and the growth-inhibiting substances inhibited it. Consequently, it was indicated that the negative geotropism of the leaf-sheath basal region in the wheat plant was controlled by synthetic growth regulators.

The geotropism of this region had no direct relation with the node and the internode, moreover it was found that there was no dorsiventrality in this region.

INTRODUCTION

THE geotropism in the nodal region of monocotyledons has been much investigated; van Overbeek (1936) observed that there was no gravitational response in the lazy type of corn plant, and Jenkin and Adair (1938) found the same in the lazy selection of rice. The photo-reaction to the geotropism of prostrate plants has been studied by Langham (1941) and Palmer (1956). It is stated by van Overbeek and Cruzado (1948) that the change from vegetative growing point to floral apex may be induced by placing the plant in a horizontal position for 3 or more days in Cabezona variety of pineapple. And it is also indicated by van Overbeek (1938) that the lower half of normal corn node contains more ether extractable auxin than the upper one, but on the contrary, in a lazy corn node the upper half contains a little excess, and the total quantity is higher in the lazy type than in the normal type. Danjo (1949) has shown that the negative geotropic movement of the first internode of wheat plant was expressed by two straight lines in 26 days of the process when the stem was lodged at the second internode.

On the basis of these facts, the author investigated the responses of the intercalary growth region of the leaf sheath to the synthetic growth regulators in an attempt to make clear the mechanism of the geotropism of the grass shoot. This report gives results of responses of leaf-sheath basal region in wheat to growth regulators with reference to the geotropism, and shows the histological changes.

MATERIALS AND METHODS

As experimental materials the author has used wheat *Triticum vulgare* (variety Nōrin No. 53) cultured in the field of the Faculty of Agriculture, Nagoya University. Stem segments containing a node were employed, which were excised from the plant when the base of the sheath of the second leaf from the uppermost leaf just below the ear has emerged from the third leaf sheath. Uniformity of material is necessary to eliminate the considerable

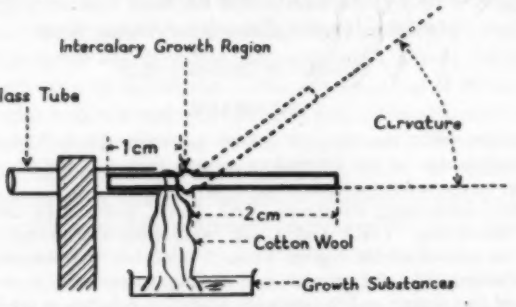


FIG. 1. The assay method of geotropism of the stem segment contained a node in wheat plant.

occasional variance, particularly with respect to the plant age, because if aged segments coexist in the materials, the value of the measurements is disturbed by lignification on the part of the intercalary growth region which is in the base of the leaf sheath.

The growth-stimulating substance and the growth-inhibiting substance in various concentrations ranging from 1 p.p.m. to 1,000 p.p.m. have been used: the former consists of indolebutyric acid (IBA), naphthaleneacetic acid (NAA), and 2:4-dichlorophenoxyacetic acid (2:4-D), and the latter consists of 2:3:5-triiodobenzoic acid (TIBA) and 2:4-dichloroanisole (DCA).

The assay method is shown in Fig. 1. The excised stem segment, cut 1 cm. below the node and 2 cm. above, is placed horizontally in a glass tube by cotton wool, and treated with the growth-regulating substances which are sucked up through the cotton wool soaked in the solution in glass dish. After incubating at 30° C. for 24 hours, the degree of negative geotropic curvature using shadowgraph and the lengths of the upper and the lower side of the intercalary growth region of the segment are measured.

Then, these intercalary growth regions were fixed in FAA, dehydrated in tertiary butyl alcohol, embedded in paraffin, cut at 5μ thick, stained by the tannic acid–iron chloride schedule of Foster (1934), counterstained with fast green, and the cell forms and length were observed with a microscope. The median longitudinal sections in the plane both including the stem axis and the upper and the lower side of the region were cut, and the cutting was done from the upper side to the lower side of the region so as to measure exactly the longitudinal length of the cells.

RESULTS

In the case of treatment with distilled water (control), it was found that the curvature was caused by the growth differences owing to stimulated growth on the lower side of the intercalary growth region, as shown in Table I (means of 10 estimates).

When treated with growth-regulating substances, IBA, NAA, 2:4-D, DCA, and TIBA, as shown in Table II, it was observed that the degree of

TABLE I

Curvature (C) and length of upper (U) and lower side (L) (mm.) in the intercalary growth region of stem segments treated with distilled water after 24 hours in darkness at 30° C.

	C	U	L
Before treatment	0	1·7	1·7
After 24 hours	21	1·8	3·2

TABLE II

Curvature (C) and length of upper (U) and lower side (L) (mm.) in the interrcalay growth region of stem segments treated with growth-stimulating substances, IBA, NAA, and 2:4-D, and growth-inhibiting substances, DCA and TIBA, at concentrations shown after 24 hours in darkness at 30° C.

	10 p.p.m.			100 p.p.m.			1,000 p.p.m.		
	C	U	L	C	U	L	C	U	L
IBA	16	2·1	3·2	3	2·8	3·2	3	2·6	2·8
NAA	13	1·9	2·9	6	2·9	3·4	5	2·2	2·7
2:4-D	13	2·1	3·0	2	3·3	3·4	2	1·8	2·1
DCA	15	1·7	2·8	12	1·8	2·5	4	1·5	2·0
TIBA	18	1·7	2·9	6	1·8	2·2	2	1·7	1·8

curvature decreased as the concentration increased. In the case of IBA, NAA, and 2:4-D treatment, marked longitudinal growth of the intercalary growth region occurred, and the elongation of both sides of this region was the maximum at 100 p.p.m. and was less to some extent at 1,000 p.p.m. than at 100 p.p.m. When treated with TIBA and DCA, the decrease of curvature was the same as for the stimulating substances but the growth of this region was on the contrary inhibited by increased concentration (see Fig. 8).

The effects of a mixture of growth stimulating substances and growth inhibiting substances, from 1 p.p.m. to 100 p.p.m. of NAA or 2:4-D with 100 p.p.m. of TIBA, are shown in Table III. As the concentration of NAA or 2:4-D was increased the degree of curvature was reduced progressively, and the growth of this region was promoted on both sides. As compared with Table II, it was clear that in the case of the mixture of 100 p.p.m. NAA with 100 p.p.m. of TIBA the growth of the region was greater than in TIBA alone, and was less than in NAA alone. With 2:4-D TIBA mixtures the same result was obtained.

TABLE III

Curvature (C) and length of upper (U) and lower side (L) (mm.) in the intercalary growth region of stem segments treated with mixtures of NAA or 2:4-D at various concentration ranging from 1 p.p.m. to 100 p.p.m. with 100 p.p.m. of TIBA after 24 hours in darkness at 30° C.

Concentration of growth-stimulating substances	1 p.p.m.			10 p.p.m.			100 p.p.m.		
	C	U	L	C	U	L	C	U	L
TIBA plus NAA	12	1·4	2·2	4	1·7	2·4	2	2·6	2·9
TIBA plus 2:4-D	10	1·6	2·4	5	2·0	2·3	5	2·9	3·3

As shown in Table IV and Figs. 2–6, according to the histological observation it was clear that in the case of control, the cell elongation occurred in the lower side, and when treated with 100 p.p.m. of 2:4-D the cell elongation was induced on both sides, but when treated with 1,000 p.p.m. of TIBA the cell elongation was inhibited on both sides (means of 20 estimates). It is indicated by this observation that the growth differences of this region are caused by the degree of increase in the cell length.

TABLE IV

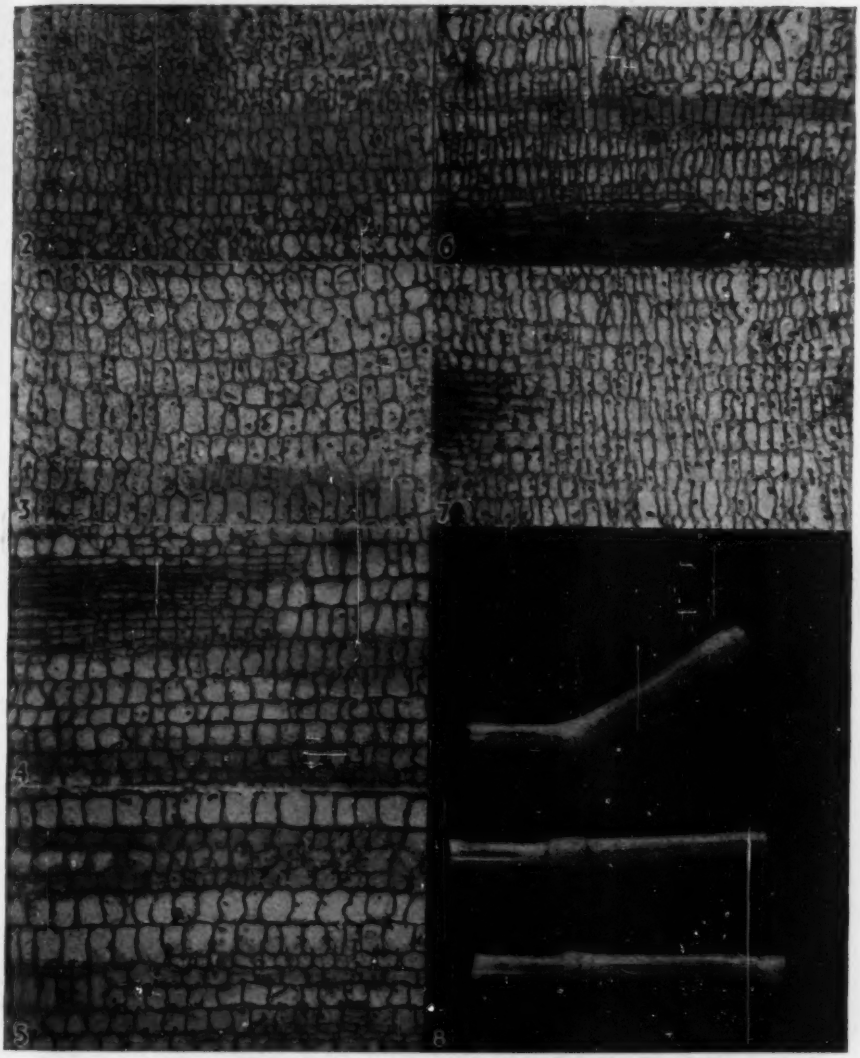
Cell length in upper and lower side of intercalary growth region of stem segments treated with distilled water (control), 100 p.p.m. of 2:4-D and 1,000 p.p.m. of TIBA after 24 hours in darkness at 30° C.

	Upper side cell length micron	Lower side cell length micron
Distilled water	31	40
2:4-D 100 p.p.m.	45	52
TIBA 1,000 p.p.m.	23	25

In consequence, the growth-stimulating substances and the growth-inhibiting substances seem to be acting in a mutually competitive manner on the intercalary growth region in the leaf sheath of wheat plant. From these facts, it may be stated that the geotropism which is caused by the relative difference of growth in the region is regulated by the activity of auxin-like substances.

DISCUSSION

The effect of TIBA in the growth process and morphogenesis have been indicated by many works. Above all, Galston (1947) showed in Avena curvature test that TIBA inhibited the action of IAA, and Thimann and Bonner (1948) obtained the same result except that the curvature in this test increased in the range where the mol ratio of TIBA and IAA was approximately 1:1. Snyder (1949) ascertained in his studies of the rooting of cuttings and the development of axillary buds of *Coleus blumei*, too, that the effect of IBA was decreased by TIBA. Kuse (1953) noticed the effect of TIBA on



FIGS. 2–8: Longitudinal sections of intercalary growth region of wheat leaf sheath after 24 hours in darkness at 30° C (×70). 2. Upper side of control. 3. Lower side of control. 4. Upper side after treatment with 2:4-D 100 p.p.m. 5. Lower side after treatment with 2:4-D. 6. Upper side after treatment with TIBA 1000 p.p.m. 7. Lower side after treatment with TIBA. 8. State of stem segments after 24 hours in darkness at 30° C (×1·4). Upper: Control. Middle: treatment with 2:4-D 100 p.p.m. Lower: Treatment with TIBA 1000 p.p.m.

geotropic curvature of petiole in his work on the effect on growth of lateral bud and tropism of petiole using *Ipomea batatas*, and described the influence of TIBA on auxin transport. On the effect of DCA, Bonner (1949) found in Avena coleoptile sections a function as an inhibitor of growth.

The facts that TIBA influences the cell elongation and tropic movement, and acts on the decrease in the effects of growth-stimulating substances are in good agreement with the author's results. Moreover, the author ascertains the interfering point of TIBA in the geotropism of wheat shoot.

From the results of the present study, it is apparent that IBA, NAA and 2:4-D promote the growth of the intercalary growth region which lies in the base of leaf sheath of wheat plant and TIBA and DCA inhibit it; these facts indicate the function as growth regulating substances. Studies of responses to mixture of growth stimulating substances in growth inhibiting substances show that NAA or 2:4-D and TIBA are mutually antagonistic in their actions on the growth of the intercalary growth region. Thus, the synthetic growth regulators used in these experiments influence in the negative geotropism of wheat plant. It is proved histologically that the difference of cell elongation between upper and lower side of the region causes geotropic curvature in the stem of wheat plant, moreover that the growth regulators act either stimulatively or inhibitively in the cell elongation of this region.

Therefore being obvious that the indicated negative geotropism depends upon the difference of cell elongation in the leaf sheath basal region in the proximity of the node, it is clear that there is no direct relation between geotropism and the node. And also the geotropism has no relation with the internode, because the internode becomes straight when the leaf sheath of a curved stem segment is removed. Furthermore it is suggested that there is no dorsiventrality in the intercalary growth region of stem segments (sheath joints) because they always curve upwards independent of the setting side when they are placed horizontally.

ACKNOWLEDGEMENTS

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Studies in Dormancy of Sycamore I. Seasonal Changes in the Growth-substance Content of the Shoot

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SUMMARY

The growth substances present in buds and leaves of sycamore (*Acer pseudoplatanus*) have been studied throughout the annual cycle, in relation to the growth and dormancy of the shoot. Extracts made with 80 per cent. methyl alcohol of the apical region and mature leaves were chromatographed and assayed with wheat coleoptile sections, lettuce seeds, cress-roots, and leaf-disks of sycamore.

An inhibitor was present in all extracts, running at approximately Rf 0·7 on chromatograms developed in isopropanol/ammonia. This was found to show various quantitative changes during the course of the year. Extracts of the apical region showed greatest inhibiting activity in the early winter, and least when active growth was taking place in May–June. There appeared to be a gradual decrease in inhibitor content of the terminal-bud during the course of the winter. During late summer and early autumn the inhibitor content of the apex increased. Extracts of the mature leaves made during summer and autumn showed that the inhibitor content of the leaves increased until late August, and then decreased to zero. This latter change is thought to be associated with the senescence of the leaves prior to abscission.

Indirect evidence suggests that the inhibitor is formed in the leaves and transported to the apex, where it accumulates. The inhibitor was found to inhibit the growth of sycamore tissue itself.

Growth-promoting activity was found on most of the chromatograms, and it was shown that this was attributable to sugar rather than auxin.

INTRODUCTION

SINCE Hemberg's papers dealing with the possible role of inhibitors in the dormancy of potato (1947) and buds of *Fraxinus* (1949), there has been renewed interest in the question of the part played by auxins and inhibitors in the control of dormancy in perennial shoots.

Eggert (1953) and Kassem (1944) concluded from their results that dormancy of buds was caused by the presence of supra-optimal concentration of auxin, which gradually diminished during the course of the winter. However, Avery, Burkholder, and Creighton (1937), Bennett and Skoog (1938), Malan (1939), and Zimmermann (1936) were of the opinion that it was a *lack* of auxin that kept buds dormant, and they found that auxin appeared only later on in the winter. With Hemberg's results indicating the possible significance of growth-inhibitors in buds of woody species, other workers directed their attention towards this type of growth-substance. Hendershot, Bailey, and Lowell (1955) and Steward and Caplin (1952) found supporting evidence

for Hemberg, for they too found a growth inhibitor present in dormant buds which decreased markedly by the end of the dormant season.

None of the workers mentioned fractionated their plant-extracts beyond an acid-neutral separation, and this therefore leads to difficulty in interpreting their results, since the growth-activity of their extracts is likely to be the sum of the promoting and inhibiting influences present. This paper describes the results of preliminary investigations into the dormancy of sycamore (*Acer pseudoplatanus*) using more refined techniques than have been employed in previous investigations, especially the use of paper chromatography to separate the various constituents of extracts.

MATERIALS AND METHODS

Plant material. All the sycamore tissues were obtained from naturally occurring stands of trees. In the case of the winter collections (Series A extracts) the apical buds were taken from the same three adjacent mature trees on each occasion, an effectively random assortment being taken from high and low branches of the three trees. The apical and leaf materials collected for the summer and autumn series (series B, C, D, and E) were from a wide range of young trees. As far as possible, uniform material was collected for each extraction.

The tissues to be extracted were always frozen at −15° C. as soon as possible after collection, and this involved a time lapse of ½–3 hours. Complete buds of the winter collection were extracted, it being extremely difficult to separate any quantity of buds into scales and primordial tissues during the early part of the winter. Extracting whole buds was considered quite justifiable in view of the fact that 'breaking' sycamore buds elongate as a whole, the scales being alive and growing along with the underlying embryonic leaves. Thus the scales themselves must have a dormancy-breaking mechanism, and it is reasonable to assume that this would be similar to that of the primordial tissues. Also, in the series A buds only, some flower-buds were included in the samples since it was impossible to differentiate with any certainty between these and vegetative buds without resorting to individual dissection of the buds. Again the assumption is made that the dormancy-breaking mechanism would be similar in both types of bud.

Series B and C extracts were obtained from exclusively vegetative buds, this being possible by virtue of the collections coming from immature trees only.

Not until the period of rapid elongation of the buds in March, was an experiment performed to compare the growth-substance content of the scales with that of the inner tissues of the bud, and this showed that there was no fundamental difference between the two with respect to inhibitor content.

The leaves extracted (Series D and E) were obtained from young trees, and only mature leaves were used.

Before being extracted, the material was always kept at −15° C.

Extraction technique. Absolute ethanol (EtOH), wet diethylether, absolute

and 80 per cent. methanol (MeOH) were compared with regard to their ability to extract all growth-active substances from tissues of sycamore. This was done in order to find a solvent which would be efficient enough to dispense with the need for a subsequent aqueous extraction, with its attendant chromatographic difficulties, and also to facilitate quantitative comparisons of growth-substances present during developmental changes of the plant. 80 per cent. MeOH appeared to be the most effective of these solvents.

The apical material to be extracted was first frozen at −15° C. and then ground up with a small amount of solid carbon dioxide. The leaves were frozen at −15° C. but not ground up with solid carbon dioxide, since extraction of leaf tissues with MeOH was satisfactory after the leaves were coarsely comminuted subsequent to being frozen. In all cases the extractions were carried out at 0° C., the flask being shaken occasionally.

Following an extraction period of generally 24–26 hours, extracts made with organic solvents were immediately filtered and, except in the case of ether, partitioned with '80/100' petrol-ether to remove chlorophylls. The dry weight of material extracted was then determined (note that in all cases 'dry wt.' refers to the dry weight after extraction).

Concentration of the extracts was carried out by distillation *in vacuo* at a bath temperature not exceeding 35° C., and finally by freeze-drying the aqueous residue down to a convenient volume for loading on the chromatographic paper.

Aqueous extracts, where made, were filtered and then made up to at least 70 per cent. ethanol to precipitate proteins present before distillation and freeze-drying.

In all the developmental studies of the apical region described in this paper, a technique of triple extraction with 80 per cent. MeOH was used, and the three extracts were combined to constitute what is simply termed, 'the 80 per cent. MeOH extract'. This method of extraction was found to remove from sycamore tissue all substances growth-active in the wheat coleoptile section test. Thus an aqueous extract made after such extraction contains no growth-active materials.

It will be noted that in these and all other extracts made with 80 per cent. MeOH, there is a considerable amount of inhibitory material that remains on the starting lines of chromatograms. It seems reasonable to ascribe this to the presence of toxic concentrations of brown resin-like materials in this region, and no attention is paid to it in the following text, especially as it shows no quantitative changes that can be correlated in any way with the state of dormancy.

In the case of the leaves, a single extraction was made with 80 per cent. MeOH, followed by absolute MeOH since the former did not penetrate the leaf tissues thoroughly—as evidenced by the leaves retaining a quantity of chlorophyll. The absolute MeOH was adjusted to 80 per cent. aqueous whilst still in contact with the leaves, and left for a further extraction period. The two extracts were combined.

The MeOH was always distilled prior to its use for extractions, or any other purpose, since the solvent as supplied contained inhibitory material. It was shown that the redistilled solvent no longer contained growth-active material.

The petrol-ether from partitions was tested to see if it had extracted any active compounds. The petrol-ether phase from a partition with an 80 per cent. MeOH extract was reduced to near dryness and shaken up several times with distilled water. The water was then concentrated by freeze-drying and chromatographed. The assay result showed that there was no significant distribution in inhibitor or promoter into the petrol-ether fraction.

80 per cent. MeOH extracts before being concentrated were stored at 0–2° C., and this period was always kept as short as possible. After reduction to the aqueous phase they were kept frozen at −15° C.

Paper-chromatography. Whatman's No. 1 paper was used throughout the work, the measured quantity of extract being strip-loaded on the starting-line by means of a 1·0 ml. graduated pipette in a current of warm air.

Chromatograms of extracts were developed, by the descending method, in the dark at room temperature, until the solvent front reached a distance of between 25 and 35 cm. from the starting-line without previous equilibration of the chromatograms. The solvent used was isopropanol/0·15 N. ammonia:: 80/20, which gave satisfactory separation of active substances contained in the extracts, and retained the brown resinous material on the starting line. After development the chromatograms were dried and examined under ultraviolet light (2537 Å), but the fluorescent pattern of a chromatogram was rarely correlated with growth activity.

Except where stated otherwise, chromatograms were cut into ten equal transverse strips, each strip being eluted in either 10 or 5 ml. of double glass-distilled water overnight at just above 0° C., immediately before the assay of the eluates.

Coleoptile section straight-growth assay. Wheat (var. 'Pilot' or 'Atle') was soaked for 2–3 hours at laboratory temperature, and then shallowly planted in washed sand, and well watered. Seeds were left to germinate and grow for three days in the dark at 25° C. and in a humid atmosphere. The subsequent technique was exactly as described by Bentley and Housley (1954), the coleoptile sections being 10 mm. in length initially.

Cress-root assay. Cress seedlings (var. 'Fine Curled') with a root length of 5 mm., were chosen from seedlings grown for 24 hours at 25° C. on damp filter-paper in 9·0 cm. diameter petri dishes, and put to grow in batches of 15 in 2·5 ml. of the inhibitor eluate on the surface of filter-paper in a 9·0 cm. diameter petri dish. The increase in root length after 18 hours growth at 25° C. was compared with that made by roots growing in water.

Lettuce germination assay. Lettuce seeds (var. 'New Market' a non light-requiring form), about 80, were planted on filter paper moistened with inhibitor eluate or water in 5·5 cm. petri dishes, and the germination level per dish recorded after 48 hours at 20–22° C.

Sycamore leaf-disk assay. The disks for the first assay (Table I) were cut

from the first pair of expanding leaves from breaking buds of a single tree on 16 March, 1957. Only leaves ranging from 3·0 to 4·0 cm. across their widest part were used. The disks for the second test (Table I) came from slightly older and larger leaves. The disks were cut with a sharp cork-borer, and had an initial diameter of 8·0 mm. Only 2 disks per leaf were taken, one from either side of the main vein. This was done in order to obtain uniform material.

TABLE I

Final diameters (mm.) of leaf-disks of sycamore 8 mm. diameter initially used as test material

Standard errors and significance of differences from water control are shown

First assay	
Dist. water	10·15±0·12
Inhibitor eluate	9·45±0·20 HS
Promoter eluate	11·90±0·08 HS
Second assay	
Dist. water	8·95±0·08
2% sucrose eluate of promoter	9·8 ±0·06 HS
2% sucrose	9·55±0·08 HS
IAA. 1·0 m./l.	9·20±0·06
IAA. 1·0 m./l.	9·15±0·08

Ten disks were floated lower-surface downwards on the surface of each inhibitor or promoter eluate, and the increase in diameter was measured after 6 days in either natural daylight or under fluorescent lights (18-hour photoperiods) in a growth cabinet at 70° F.

EXPERIMENTAL

Growth-substance changes were traced from early May, when the twigs were actively elongating, until early April of the following year when the apical winter-buds were about to 'break' for that year's flush of growth.

It was considered necessary to bioassay as many chromatograms of extracts at one time as possible, in order to avoid variations in the test from occasion to occasion. Extracts were grouped into 5 series, each of which was bioassayed all together: series A from 31 October until 3 April 1955–6 (6 extracts of buds); series B between 3 May and 26 June 1956 (3 bud extracts), and series C from 26 June until 31 October 1956 (8 bud extracts); leaf extracts (series D and E) ranged from early May to mid-October 1956 (11 extracts)—3 in series D and 8 in series E).

Bud extracts. These were all made using the technique described in the relevant section of this paper. It will be seen from Fig. 1 that there are changes in the dry weight: fresh weight ratio during the year and for this reason quantities of extracts of the same equivalent dry weight only were used for comparison.

Series A (October–April). After concentration, the equivalent of 1·0 g. dry wt. of each extract was chromatographed and assayed (Fig. 2). In this case

each of the 10 chromatogram segments was eluted in 10 ml. of water. The results show the presence of an inhibitor in all the extracts, running at about Rf 0·6–0·8 and tailing badly on most of the chromatograms. Also, in all but the first and last of the series there was a promoter occurring at Rf 0·2–0·4.

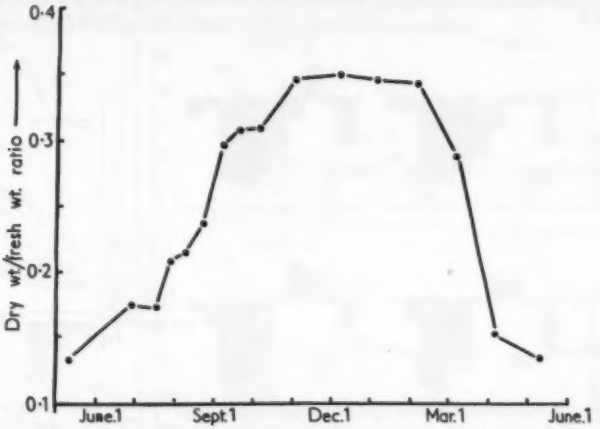


FIG. 1. Changes in the water-content of the terminal-bud during the year, as measured by the changes in the dry wt.: fresh wt. ratio. The first signs of swelling of the buds were visible on 3 February, and by 3 April the buds had burst.

On 31 October (Fig. 2A), a high level of inhibitor was present, and promoter, if present, was masked by the tailing of the inhibitor. By 5 December (Fig. 2B) the promoter had appeared, or become unmasked, and the inhibitor decreased slightly. In the extracts made in January and February (Figs. 2C and 2D) these changes were further pronounced, there being slightly more promoter, and less inhibitor, present in February than in January. However, by 5 March (Fig. 2E) the promoter had decreased, though the inhibitor had continued its original trend by decreasing still further. In the final sample on 3 April (Fig. 2F) the promoter had disappeared, and the inhibitor decreased to a minimum for this series.

A further series of chromatograms of these same extracts was developed (using the equivalent of 1·0 g. dry wt. in each case), only 2 zones of which were taken to be eluted and assayed, viz. Rf 0·1–0·4 and Rf 0·4–0·8. The first zone (promoter) was eluted in 30 ml. of water, and the second (inhibitor) in 40 ml. The results of assays are seen in Fig. 3, each point being the mean for 10 coleoptile section replicates. It is apparent that the promoter (curve A) does increase up to February and then falls away until at bud-break there is a negligible quantity present. The inhibitor shows, at a dilution of $\times 5$ (curve C, Fig. 3), a clear and steady decrease throughout the course of the winter and spring. The reason for the irregular curve B is probably due to the inhibitor concentration exceeding the highest level allowing a quantitative response by coleoptile sections, except perhaps in the extract of 3 April.

In order to check these results a fresh series of 6 extracts was prepared of buds collected on the same dates as for the first series. The equivalent of 0·2 g. dry wt. in each case was chromatographed, since the foregoing results

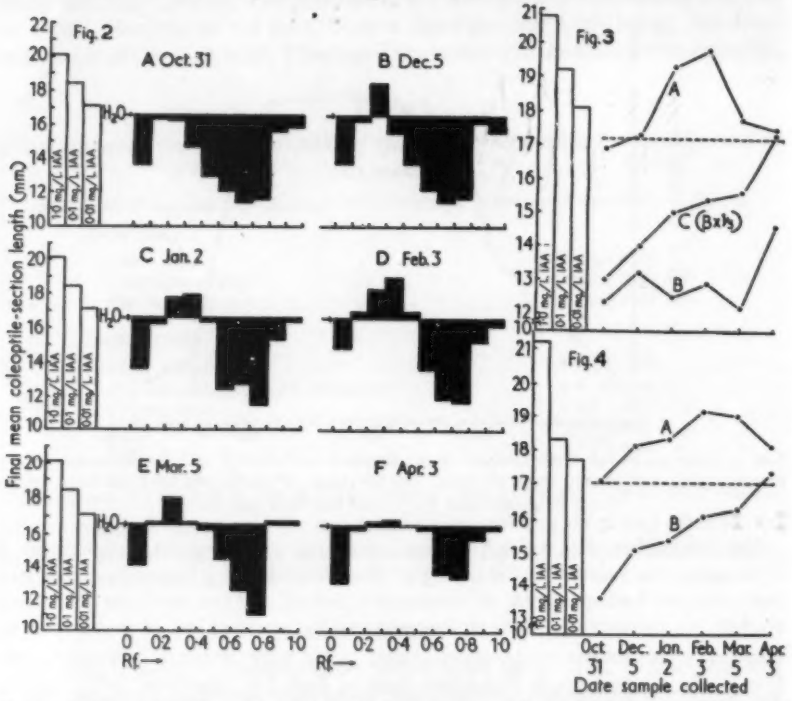


FIG. 2. Wheat coleoptile-section assay of chromatograms of 1·0 g. dry wt. equivalents of extracts obtained from winter terminal-buds.

FIGS. 3 and 4. Wheat coleoptile-section assay of promoting (A) and inhibiting (B and C) eluates from chromatograms of 1·0 g. dry wt. equivalents of extracts of winter terminal-buds in Fig. 3 and 0·1 g. equivalents in Fig. 4.

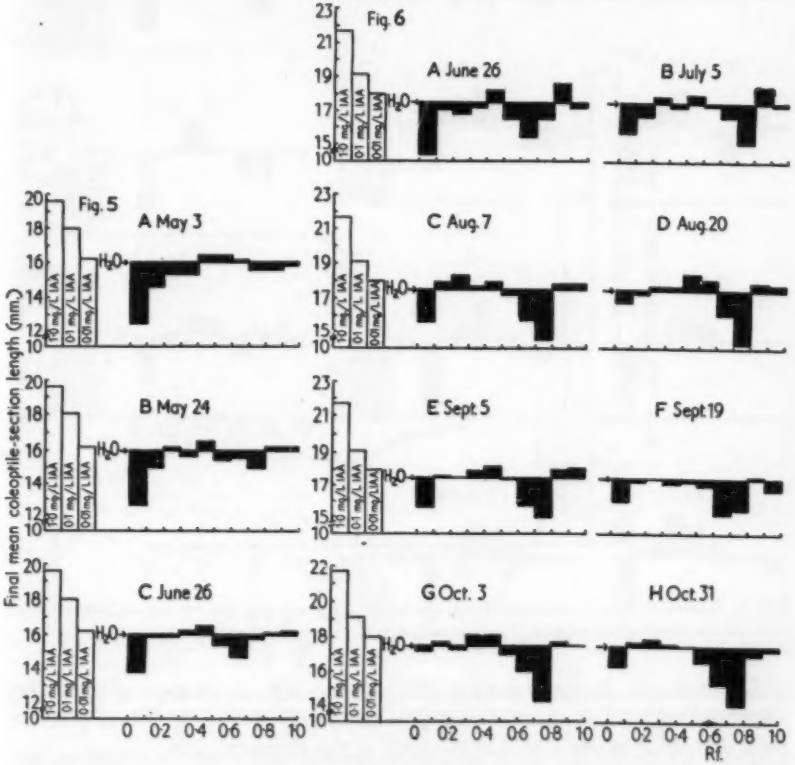
indicated that the chromatograms were overloaded at 1·0 g. Furthermore, the chromatograms after development were each halved vertically; one series of halves was assayed and the other was stored at −15° C. until the results of chromatography had been ascertained. (Results were essentially similar to those given in Fig. 2 and are not published here in order to save space.) Then the promoter and inhibitor zones Rf 0·2–0·5 and 0·5–0·8 were assayed (Fig. 4).

The results substantiate those from the first series illustrated in Figs. 2 and 3; that is, the inhibitor decreases during the winter and spring and the promoter increases until February and then falls away.

Series B extracts (May–June). The extracts of 'apices' in this series and series C, were obtained from the tissues of the apical region itself plus the

very small (up to 0·5 cm. in length) expanding leaves and the uppermost, non-elongated, internode.

The equivalent of 0·1 g. dry wt. of each of the 3 concentrated extracts was chromatographed and assayed with coleoptile sections (Fig. 5). It was found that there was a small progressive increase in inhibitor content in this period of the year. There was very little indication of promoter.



FIGS. 5 and 6. Chromatograms of extracts of the apical region assayed with wheat coleoptile sections, the equivalent of 0·1 g. dry wt. having been chromatographed in each case. On 3 and 24 May the shoots were actively growing, but on 26 June extension-growth had ceased, but bud development had not commenced. The first signs of bud scales were visible on 25 July, and bud development was complete by 5 September.

Series C extracts (June–October). The equivalents of 0·2 g. dry wt. of each extract were chromatographed. The developed chromatograms were divided in a similar manner to that used for the 'repeat' extracts of series A, 0·1 g. dry wt. equivalent therefore being on each half. The results of the assays of the first series of halves are in Fig. 6. They show the presence of the same inhibitor as in series A and B, and there is more inhibitor in the last extract

of the series (Fig. 6H) than in the first (Fig. 6A). The intermediate extracts show, in general, a progressive increase in inhibitor content up to August, but thereafter the concentration remained fairly constant.

The other halves of the chromatograms had the inhibitory zones (Rf 0·5–0·8) cut out and assayed (Fig. 7), which shows that the inhibition of the apical region increased up to 20 August and then remained reasonably steady. There was little indication of promoting substances.

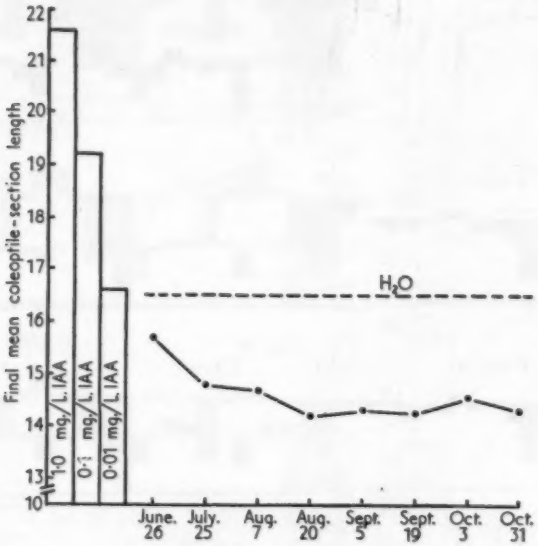
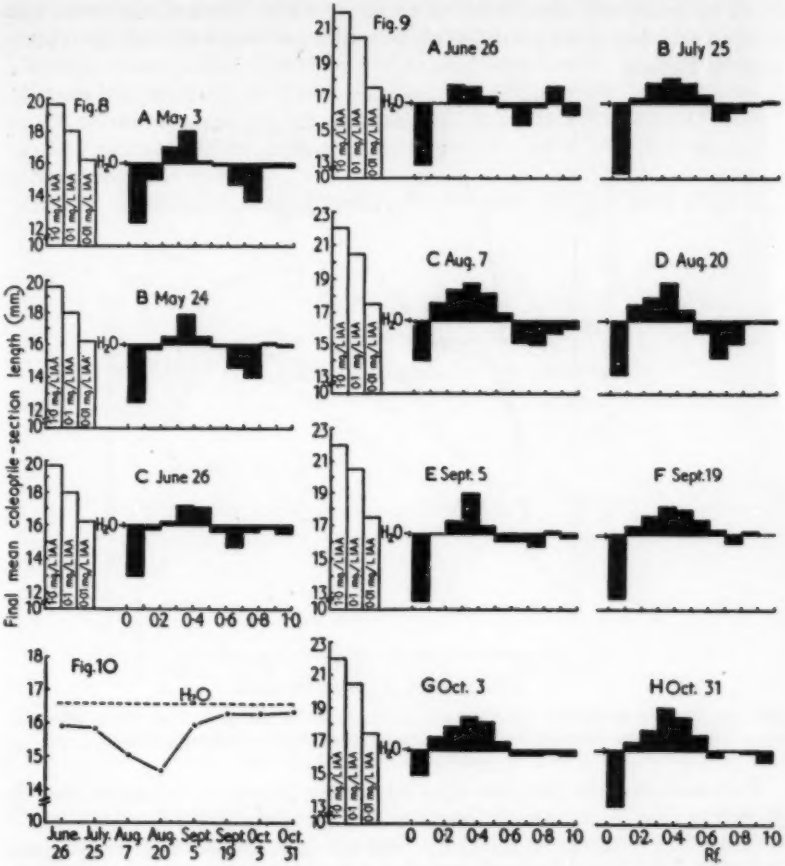


FIG. 7. Inhibitory eluates obtained from chromatograms of 0·1 g. dry wt. equivalents of series C extracts (apical region, June–October), assayed with wheat coleoptile-sections.

Leaf extracts. Series D extracts (May–June). The equivalent of 0·5 g. dry wt. of each extract was chromatographed and assayed (Fig. 8). There was very little difference in the inhibitor contents of the three extracts.

Series E extracts (June–October). Once again the equivalent of 0·5 g. dry wt. of each of the extracts was chromatographed and assayed (Fig. 9); the inhibitory regions of a similar series were assayed (Fig. 10). Both these figures show that the inhibitor content of the leaves increases until 20 August, and then falls off to virtually zero by 19 September. The promoter content remained high right to the end of the series.

Inhibitor activity assayed with lettuce seeds and cress roots. The inhibitor has been found to inhibit the growth of cress roots, and the germination of lettuce seeds, as well as the growth of wheat coleoptiles. The sensitivity of the lettuce-seed germination test to the inhibitor has been found to be much lower than either of the other two tests, but this may be due to the testa acting



FIGS. 8 and 9. 0·5 g. dry wt. equivalents of series D extracts of mature leaves (May–June) and series E extracts of mature leaves (June–October). Yellowing of the leaves had commenced on 19 September and by 17 October leaf-abscission was occurring.

FIG. 10. Inhibitory eluates from chromatographed extracts (0·5 g. dry wt. equivalents) of mature leaves, assayed with wheat coleoptile sections.

as a barrier between the embryo and the inhibitor solution. Germination was defined as being the emergence, no matter how little, of the radicle.

At no dilution does the inhibitor promote the growth of coleoptile sections or cress roots (Activity curves, Fig. 11).

Effect of the inhibitor and promoter on the growth of sycamore leaf-disks. This experiment was performed in order to observe the effect of the inhibitor and promoter on the growth of sycamore tissues.

The equivalent of 0·35 g. dry wt. of the extract of buds made on 5 December 1955 was chromatographed, and the regions Rf 0·55–0·8 (inhibitor) and Rf

0·2–0·45 (promoter) were eluted in 10 ml. of water. These eluates were then assayed with leaf-disks according to the technique described, and the result is seen in Table I.

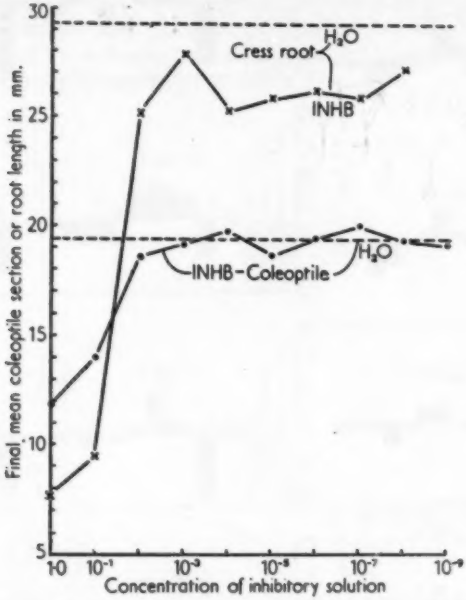


FIG. 11. Activity curves for the inhibitor, as measured by the growth of wheat coleoptile sections and cress roots in × 10 dilutions of the inhibitory eluate.

It is seen that the inhibitor does inhibit the growth of sycamore, and the promoter clearly increases the expansion of the disks.

The effect of the promoter in the presence of sugar was then investigated, in order to find out if this is a specific leaf-expansion factor, or whether the result obtained could be attributed to the presence of sugars and perhaps other nutrients in this region of the chromatogram (see spray reactions following). Thus, an identical chromatogram of the extract made on 5 December had the promoting zone removed and eluted in 2 per cent. 'analar' sucrose solution, and the leaf-disk growth in this was compared with that in water and in 2 per cent. sucrose. The result (Table I) certainly suggests that the promoting activity of this region of the chromatogram may be due to sugar.

On the basis of this result, it was decided to investigate further the reactions of the 'promoter' and sucrose in the coleoptile-section test. Thus a comparison of the promoter with 3-indoleacetic acid (IAA), in relation to their interactions with sucrose, was carried out.

The equivalent of 2·5 g. dry wt. of the extract of mature leaves made on 3 May 1957 which gave considerable promotion between Rf 0·2–0·4 with

0·5 g. dry wt., was chromatographed and gave a very positive reaction for sugar in this region. The zone Rf 0·25–0·4 was eluted in 20 ml. of water and the following wheat ('Atle') coleoptile-section assays made with: (*a*) 5 ml. sucrose solutions at concentrations from 0 to 20 per cent.; (*b*) promoter eluate 2·5 ml.+ 2·5 ml. sucrose 0·0, 1·0, 2·0, 4·0, 10·0, and 20·0 per cent.; (*c*) 2·5 ml. of IAA (0·1 mg./l.)+sucrose as in (*b*); (*d*) 2·5 ml. promoter+2·5 ml. of IAA (0·1 mg./l.); (*e*) water control.

Several significant facts emerged from the result of this assay (Fig. 12).

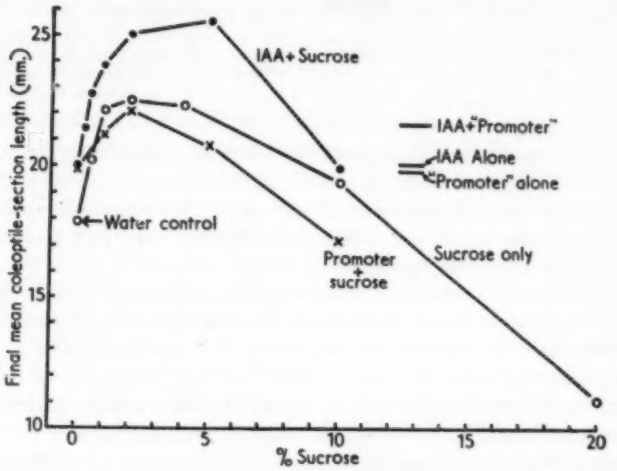


FIG. 12. Interaction of the growth-promoter, sucrose and 3-indoleacetic acid (IAA) in the wheat coleoptile-section assay.

There was no synergism between the promoter and sucrose at any concentration of the latter, whereas IAA showed very marked synergism with sucrose. IAA+the promoter gave a promotion greater than was obtained with either of them alone. As little as 0·25 per cent. sucrose gave promotion as great as that achieved by 0·1 mg./l. IAA or the promoter eluate. 10 per cent. sucrose with the promoter eluate showed inhibition possibly caused by osmotic effects.

Spray-reactions of chromatograms of series A extracts. The sprays used were *p*-dimethylaminobenzaldehyde (for indole compounds), HNO_2/HNO_3 (also for indole compounds), and aniline hydrogen-phthalate (sugars). Only with the last named (sugar) spray was any colour reaction obtained (Fig. 13). The loading of the spots for spray-reactions was of the same order as that of the chromatograms loaded with the equivalent of 1·0 g. dry wt. (Fig. 2), and therefore the distribution of sugar is sharply correlated with the promoting zone on the chromatograms. Also, as mentioned in the last column of Fig. 13, the quantity of sugar present (as judged by the intensity of the brown colour spot) shows a strong relationship to the changes in the amount of promotion

obtained with the different extracts; thus the colour was most intense with the extract made on 3 February, and least with those extracts made on 31 October and 3 April.

FIG. 13. Result of spraying chromatograms of extracts of winter terminal-buds with aniline hydrogen-phthalate. Rf of brown band and intensity of coloration are indicated.

DISCUSSION

The foregoing results indicate that the concentration of an inhibitor present in all extracts of sycamore buds and shoot apices shows a very positive correlation with the state of dormancy of the tissues. Thus there is most inhibitor present when the tissues are most dormant (October), and least when the apex is actively growing (April–May).

It has been shown previously (Wareing, 1954) that in sycamore, the photoperiodic responses at the shoot apex are determined by the day-length conditions to which the mature *leaves* are exposed. The production of an inhibitor by mature leaves under short day conditions was postulated to account for the cessation of growth of the shoot apex. From the results presented here, and others dealing with the photoperiodically induced inhibitor changes (in the press), it is apparent that the inhibitor content of the leaves increases with decreasing length of day under natural conditions until late August. It is significant that after this time the inhibitor concentration falls off, and this is probably associated with the senescence of the leaves, for the appearance of the leaves from 5 September onwards is suggestive of a gradual cessation of metabolic activity, leading to abscission in late October. The extracts of leaves were made in 1956, the summer of which was extremely cool and cloudy; this may mean that the senescence of the leaves commenced earlier than is usual. The absence of the inhibitor from senescent leaves would seem to indicate that the inhibitor is not simply a toxic substance present in non-living material, but is produced by actively metabolizing cells. Several observations suggest that the inhibitor is produced in the leaves and transported to the apex. The increase in inhibitor content of the leaves up to 20 August is paralleled by a similar increase in that of the apex, which does not increase after this date, and decreases only after being exposed, in a leafless condition, to winter conditions. The concentration of inhibitor in the leaves for any date is less than a fifth of that in the apical tissues for the same date, except perhaps where active shoot elongation is taking place in May (e.g. in Fig. 6 for series B and Fig. 7 for series C extracts the equivalent of 0·10 g. dry wt. of each extract was used, compared with 0·5 g. dry wt. of series D and series E

extracts (Fig. 9 and 10), in order to obtain similar levels of inhibition by the apical and leaf extracts). These observations suggest that the inhibitor is produced in the leaves and is transported to the shoot apex, where it accumulates.

The question arises as to whether the changes in inhibitor content play a primary role in controlling the onset of and emergence from dormancy, or whether the variations in inhibitor level represent secondary effects of changes in the state of dormancy. The results presented here shed little light on this problem. It is significant that the inhibitor does inhibit the growth of sycamore leaf disks. It is possible, however, that under normal conditions the distribution of the inhibitor in the cell is strictly localized and that it does not have access to the growth-regulating sites.

The changes noted in the promoter present in some extracts may also be of some significance, especially in the case of the series A extracts. However, the results of the experiments to investigate the possibility that sugars are the cause of this promotion apparently reduce the importance of this factor. The conversion of starch reserves to sugar, and the utilization of the latter by the growing bud, would explain the build-up in sugar concentration from October–February (see Figs. 2D–F, 9D–F, and Fig. 13), and its subsequent reduction. It is only when the leaves have expanded and are therefore synthesizing new sugars, that promoting activity can again be observed in chromatograms of extracts of leaves. It is possible that production of sugars during the winter is responsible for the observed increased water-content of the buds during this period (p. 354), due to either osmotic or non-osmotic mechanisms. The effects of sugars in plant extracts upon the growth of coleoptile sections has been dealt with at greater length by Booth (unpublished) working in these laboratories.

In conclusion we can state that in *A. pseudoplatanus* there occurs a growth inhibitor which possibly controls the state of dormancy in the shoot apex. This inhibitor never disappears from the apical region at any time of the year, but it does show very striking quantitative changes. The inhibitor also occurs in all other tissues of sycamore that have been extracted (besides those mentioned in this paper we can add young leaves, stems, and seed embryos), and the evidence suggests that it is produced in the leaves, especially under the influence of short days, and is transported to the apex where it accumulates, to decrease only during the winter.

The decrease in inhibitor content of the apex during the winter is possibly due to the effect of chilling, though more work is to be done to establish the relationship between chilling and the inhibitor.

No evidence of auxin was found in any of the extracts, though this was possibly due to the low equivalent weights of materials assayed.

Thus, assuming that in the intact sycamore plant the inhibitor is free to come into contact with the growth-controlling centres, it would seem justifiable to conclude that the quantitative changes noted will have an effect upon the growth of the plant. In fact, growth in sycamore may be controlled by a balance between this inhibitor and an unknown auxin, or perhaps between

the inhibitor and another type of growth-promoting substance not detectable by the wheat coleoptile-section assay.

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Electric Potential Differences and the Donnan Equilibrium in Plant Tissues

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SUMMARY

The effect of various factors on the change of the potential difference between plant tissues and the external solution of electrolyte with change of concentration of the latter is considered. The effect of the presence of calcium ions in the free space when the external electrolyte is potassium chloride is reported and discussed.

INTRODUCTION

MOST writers now agree that the changes in electric potential difference (P.D.) between a piece of plant tissue and an electrolyte in contact with it when the concentration of the latter is changed are dependent on the presence of indiffusible anions in the tissue (a Donnan system). On this assumption, some of the parameters of the Donnan system have been deduced. Such parameters are the concentration (A) of the indiffusible anions (Meyer and Sievers, 1936; Tendeloo, Vervelde, and Zwart Voorspuy, 1946), the pK of the acids which give rise to the anionic fixed charges at ordinary external pH values (Lundegårdh, 1941; Tendeloo, Vervelde, and Zwart Voorspuy, 1946), and the isoelectric point of the amphoteric molecules thought to comprise the Donnan system (Lundegårdh, 1941; Hope, 1951; Bange, 1957). The P.D. was originally interpreted by Lundegårdh (1941) as an 'adsorption-exchange potential' and later, wrongly, by Hope (1951) as a diffusion potential.

A number of factors make difficult the precise interpretation of the results of experiments on change of P.D. Some of these factors are as follows:

1. At dilute external concentrations, the P.D. is sensitive to small changes of external pH (caused, for example, by evolved CO_2) and increasingly so the more dilute the solution and the higher the pK of the acids producing the indiffusible anions. This is probably the explanation of the P.D. changes in stationary solutions obtained by Hope and Stevens (1952) and used by them as supporting evidence that the initial uptake into the free space was mainly by diffusion.

2. At constant external pH the internal pH and hence the concentration A will rise as the external concentration of electrolyte C is increased. In consequence, the change in P.D. for a tenfold change in C is less than that calculated on the assumption that A is constant. This point was considered

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by Tendeloo, Vervelde, and Zwart Voorspuy (1946) and in more detail by Vervelde and Tendeloo (1953), but not by Bange (1957).

3. If the counterions in the Donnan system are not all of the same valency as the cations in the external solution the P.D. is a different function of C from that when all the cations are alike.

4. The volume of the Donnan system may change, with consequent change in A, as C changes. If the volume increases with C, as it should if the total volume of the cell remains constant, then the change of P.D. would be increased.

5. As the external concentration is increased the P.D. will be increasingly reduced by current flowing through parallel, water-filled paths: the 'Water Free Space' (W.F.S.) of Briggs, Hope, and Pitman (1958).

Some of the consequences of neglect of these factors are discussed by Briggs and Robertson (1957) and in more detail by Briggs (1957). The purpose of the present paper is to give the results of experiments made to measure the P.D. changes as a function of external concentration with the above factors controlled as far as possible or taken into account in the interpretation. The results of these experiments are compared with published results and the discussion directed to an attempt to assess the reliability of the values for A and the pK of the acid HA in the D.F.S., derived from such results.

EXPERIMENTAL METHOD

Disks 1 mm. thick and 20 mm. in diameter were cut from the roots of *Beta vulgaris* and placed in aerated distilled water for about 4 days and then, for most experiments, into a solution of 10 m. equiv./l. KCl for a further 4 days at least. Roots, about 5 cm. long, excised from *Vicia faba*, were put into distilled water for at least 1 day and then into KCl for a minimum of 1 day. The disks were mounted vertically between two perspex compartments, the edges being sealed with vaseline. The experimental solutions were led out from the bases of the two sides where electrical contact was made with them by means of two tubes of polythene, containing saturated KCl/2 per cent. agar (see Fig. 1*a*). Measurements of P.D. were made with bean-root segments in a similar arrangement except that the base of the segment was sealed into a hole in a perspex chamber containing slowly running 0·1 m. equiv./l. KCl and the tip of the root dipped into a small polythene tube (see Fig. 1*b*).

The P.D.'s were measured with a pH electrometer connected to a pen recorder on the chart of which a deflexion of 1·3 mm. represented 1 mV. Calomel half-cells were used to connect the electrometer to the saturated KCl, into which dipped the KCl/agar tubes. Thus, all liquid-junction potentials should have been small. The temperature was between 17 and 22° C., but did not vary more than 0·5° C. in an individual experiment.

RESULTS

P.D. as a function of C in tissue pretreated with KCl. Disks and root segments, after the preparation described above, were treated with KCl solutions

of concentration 50 m. equiv./l. for three periods of 30 min. The volume ratio, solution to tissue, was about 30:1. Previous experience with beet disks has shown that with this treatment practically all the counterions in the free space will be replaced by K ions (see Briggs, Hope, and Pitman, 1958). The free space was then brought to equilibrium with 0·1 m. equiv./l. KCl by four 10-min. treatments with this solution. Then one side of the disk, or with the roots, the basal end, was kept at a constant concentration of 0·1 m.

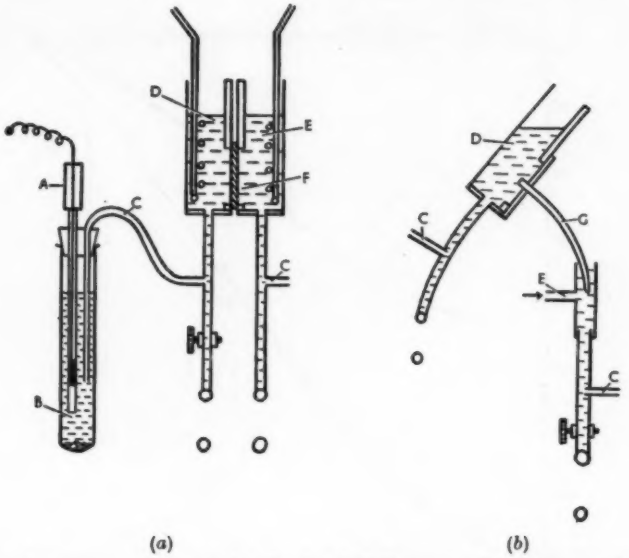


FIG. 1. Arrangement for measuring the electric P.D. across a disk of beet root (*a*) or along a bean root (*b*). See also text. *A* Calomel half-cell. *B* Saturated KCl. *C* Saturated KCl-agar tubes. *D* 0·1 m. equiv./l. KCl. *E* KCl solution of varied concentration C_e. *F* Beet disk. *G* Bean root segment.

equiv./l. by running an aerated solution slowly through the compartment, while the other locus was brought into contact with increasing concentrations of KCl, the increases being in the ratio of 3·16:1, and the P.D. measured at each concentration.

The results for beet disks are shown in Fig. 2 (*a*). The P.D. has the sign of the stronger solution relative to the fixed 0·1 m. equiv./l. side and is plotted against the logarithm of the external concentration.[1] The curve for root segments is similar.

In these experiments the P.D. was steady and repeatable, the standard error of the mean of five experiments being usually less than 2 mV.

[1] The P.D. when both loci are in contact with 0·1 m. equiv./l. KCl is taken as an arbitrary zero. With beet disks this P.D. is in fact zero, but with the root segments it is of varying sign and magnitude, depending on the length of the segment and other factors.

P.D. as a function of C after treatment with $CaCl_2$. After measurements of the P.D. resulting from KCl pretreatment, the tissue was placed in $CaCl_2$ solution, 20 m. equiv./l., for three periods of 40–50 min. to replace the K ions in the Donnan Free Space (D.F.S) by Ca (see Briggs, Hope, and Pitman, 1958). $CaCl_2$ was then removed from the W.F.S. by sufficient rinsing with distilled water. After this treatment the P.D. varied with KCl concentration as shown in Fig. 2(*b*). Again, with bean roots, the results were similar. The

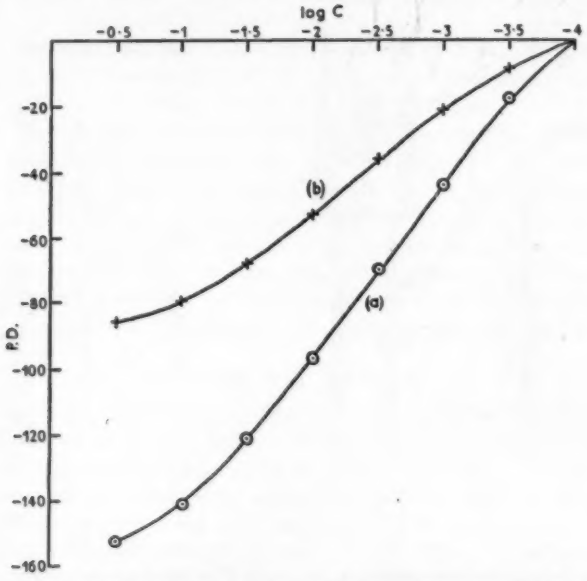


FIG. 2. Change of P.D. in mV with change of concentration in equiv./l. of KCl. (*a*) After replacement of bivalent cations of the free space by K ions. (*b*) After substitution of Ca ions for K ions in the free space.

tissue had been in contact with 316 m. equiv./l. KCl solution in their previous measurements but this contact lasted only about 20 sec. That this concentration, high enough to cause plasmolysis, is not responsible for the much smaller slope of the P.D. curve as compared with that for the tissue before treatment with $CaCl_2$ is shown by the fact that a similar curve to that in Fig. 2 (*a*) was obtained after yet further treatment with 50 m. equiv./l. KCl as above.

The curve for tissue without the special pretreatment to establish a known valency of cation in the D.F.S. was found to lie between the curves in Figs. 2 (*a*) and (*b*).

DISCUSSION

Since the change of P.D. for a given change of concentration of KCl is less the smaller A, the concentration of indiffusible anions in the D.F.S., and since the steps taken to remove diffusible bivalent cations from the D.F.S. result in

the expected increase in this change of P.D. it follows that earlier estimates of A from the results of experiments where these steps were not taken are likely to be too low.

In our experiments where these steps were taken the effect of evolved carbon dioxide on pH was reduced by using flowing solutions. To have reduced it yet further by use of a lower temperature, as did Bange (1957), would have been a further improvement.

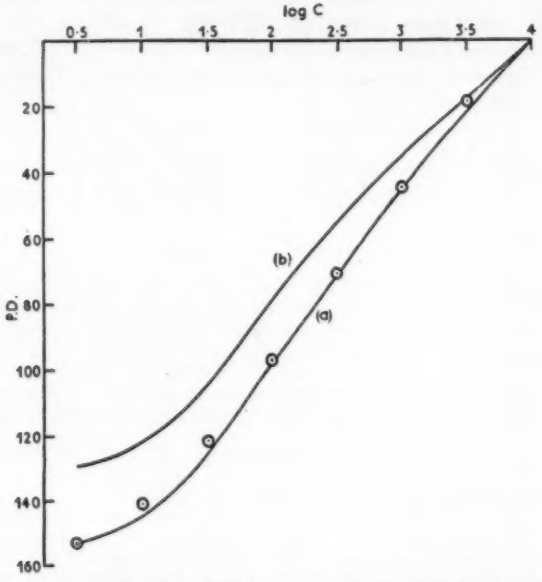


FIG. 3. Calculated changes of P.D. with change of concentration when $a = 100$ m. equiv./l. (*a*) pK = 3, (*b*) pK = 4, ⊙ from Fig. 2(*a*).

Neglecting the other complications we could proceed as follows. Tendeloo, Vervelde, and Zwart Voorspuy (1946) have shown that in a Donnan equilibrium between indiffusible anions, mobile K, H, and Cl ions on the one side and KCl and HCl on the other, the Donnan ratio r is given by:

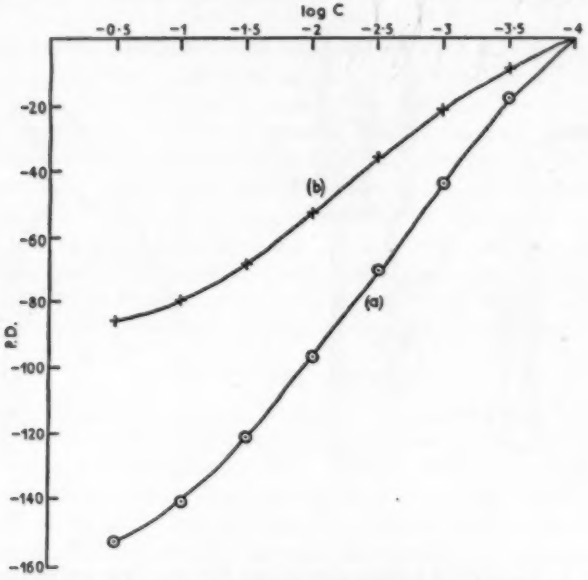
$$r^3+r^2\left(\frac{a}{C_1+C_2}+\frac{C_2}{k}\right)-r-\frac{C_2}{k}=0 \qquad (1)$$

where a is a constant $= A+HA$; C_1 is the KCl concentration and C_2 the HCl concentration outside; k is the dissociation constant of the acid HA which has a concentration HA. A as before is the concentration of indiffusible anion. The Donnan P.D. is then given by:

$$E = RT/F \ln r. \qquad (2)$$

The external pH in the present experiments was about 5·7. From a comparison of experimental results with those calculated for assumed values of a and k

P.D. as a function of C after treatment with $CaCl_2$. After measurements of the P.D. resulting from KCl pretreatment, the tissue was placed in $CaCl_2$ solution, 20 m. equiv./l., for three periods of 40–50 min. to replace the K ions in the Donnan Free Space (D.F.S) by Ca (see Briggs, Hope, and Pitman, 1958). $CaCl_2$ was then removed from the W.F.S. by sufficient rinsing with distilled water. After this treatment the P.D. varied with KCl concentration as shown in Fig. 2(*b*). Again, with bean roots, the results were similar. The



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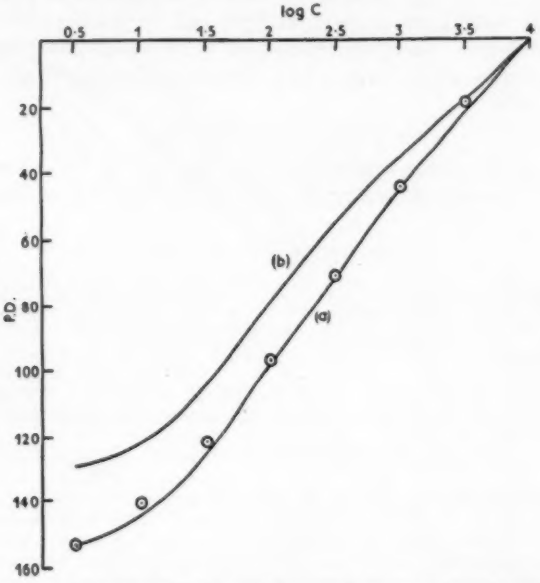

FIG. 3. Calculated changes of P.D. with change of concentration when a = 100 m. equiv./l. (*a*) pK = 3, (*b*) pK = 4, ⊙ from Fig. 2(*a*).

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$$r^3+r^2\left(\frac{a}{C_1+C_2}+\frac{C_2}{k}\right)-r-\frac{C_2}{k}=0 \qquad (1)$$

where a is a constant $= A+HA$; C_1 is the KCl concentration and C_2 the HCl concentration outside; k is the dissociation constant of the acid HA which has a concentration HA. A as before is the concentration of indiffusible anion. The Donnan P.D. is then given by:

$$E = RT/F \ln r. \qquad (2)$$

The external pH in the present experiments was about 5·7. From a comparison of experimental results with those calculated for assumed values of a and k

as a function of C_1, the appropriate values of a and k can be estimated. Fig. 3 shows the curves for P.D. so calculated putting $a = 100$ m. equiv./l. and $k = 10^{-3}$ and 10^{-4} (pK = 3 and 4). On this basis the data for beet disks are apparently consistent with a pK of 3 and a about 100 m. equiv./l. The above analysis neglects both possible diffusion potentials due to differential mobilities of K and Cl ions in the D.F.S., about which there is no information. It neglects short circuiting and it also treats a as invariant with concentration:

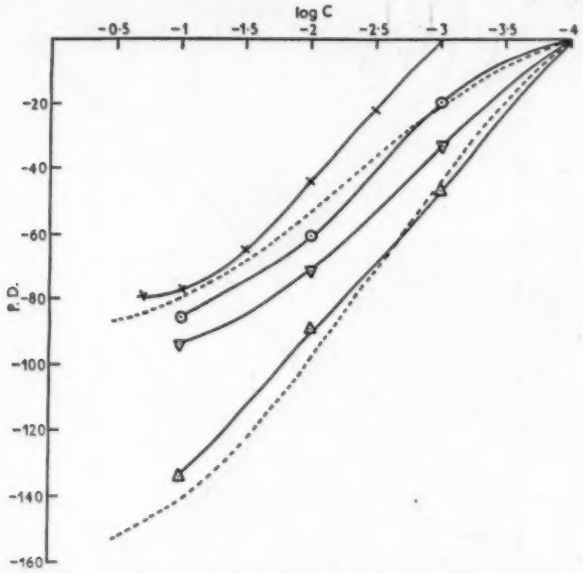

FIG. 4. Changes of P.D. with change of concentration of KCl. × slices of potato tissue from Bange (1957). ⊙ bean roots from Hope (1951). ▽ bean roots from Tendeloo *et al.* (1946). △ wheat roots from Lundegårdh (1941). - - - slices of beet tissue from Fig. 2.

it would vary with C if the volume of the D.F.S. is a function of C (Briggs, 1957).

Therefore it is not surprising that a estimated by the above procedure is different from the value, 560 m. equiv./l., calculated by Briggs, Hope, and Pitman (1958) from an analysis of the exchange of cations. The effect of short circuiting, &c., should, however, be negligible when C_1 is less than about 1 m. equiv./l., so that the value for the pK is probably reliable. Moreover, it agrees with that obtained by measuring the number of exchange sites in the D.F.S. as a function of pH (Hope, unpublished); from the pH giving half maximum ionization the pK can be calculated, the result being about 3 (see also Briggs, Hope, and Pitman, 1958).

Fig. 4 shows the results of Bange (1957) for the P.D. across slices of potato tuber, transposed to show the P.D. change from 1·0 to 190 m. equiv./l. of

KCl, and also adjusted to 20° C. The curve falls between those of Figs. 2 (*a*) and 2 (*b*) of the present paper, which means, unless potato tissue is very different from beet, that the former probably contained some bivalent cation in the D.F.S. at the time of measurement. This is supported by Bange's finding that pre-immersion of the potato tissue in 60 m. equiv./l. KCl for some days before the experiment raised the P.D. for a 3 : 1 concentration ratio. Hence Bange's estimate of 66 m. equiv./l. for A is too low since it assumes that only univalent cations are present in the D.F.S.

Similarly the results of Lundegårdh (1941), and those of Tendeloo, Vervelde, and Zwart Voorspuy (1946), and Hope (1951), also shown in Fig. 4, probably also lead to an underestimation of A and to an overestimation of pK.

ACKNOWLEDGEMENT

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Factors affecting the Abscission of Reproductive Organs in Yellow Lupins (*Lupinus luteus* L.)

II. THE EFFECTS OF GROWTH SUBSTANCES, DEFOLIATION, AND REMOVAL OF LATERAL GROWTH

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SUMMARY

Abscission of flowers in *Lupinus luteus* L. (var. Weiko II) without growth of ovaries is followed by abscission of small pods (15–20 mm. long). Normally flower abscission is much more pronounced than pod abscission.

Abscission was delayed on plants from which laterals or their terminal and axilliary buds were removed. Flower abscission was not affected, but pod abscission increased as a result of defoliation.

When flowers at the base of the main inflorescence were replaced by auxins and anti-auxins flower abscission was induced in either an auxin pattern in which most of the flowers near the site of application dropped, and pods developed on the apical whorls, or an anti-auxin pattern in which pods developed on basal whorls near the site of application but not higher up. The anti-auxin pattern was similar to the pattern of abscission normally induced by developing pods on basal whorls.

α-Naphthylacetic acid (NAA) was much more effective in inducing abscission than β-indolylacetic acid (IAA). 2:3:5-triiodobenzoic acid (TIBA), NAA, and IAA applied in mixtures at various concentrations acted mainly antagonistically, i.e. the abscission-inducing effect of NAA and IAA was depressed in basal whorls, and in apical whorls the effect of TIBA was less prevalent. Consequently the effect of the mixtures on the total number of pods was about equal to that of the most active component by itself.

All growth substances seemed to move much more efficiently in acropetal direction than in basipetal direction in the flower stalk. Transport in lateral direction was very limited.

The effect of growth substances applied on laterals was enhanced by defoliating the main stem.

The influence of assimilates on flower and pod abscission and transport of growth substances is discussed.

INTRODUCTION

IT has been shown that developing pods in lupins (*Lupinus luteus* L., var. Weiko II) induce abscission of flowers on the more apical whorls of the same inflorescence (Van Steveninck, 1957). Further it will be shown that abscission of flowers and fruits is also highly dependent on growth and development of

basal and apical laterals. In connexion with existing theories of abscission (reviewed by Addicott and Lynch, 1955) it would be of interest to ascertain whether this dependence was due mainly to nutritional competition or to a hormonal mechanism. In the latter case auxin is considered to be the main co-ordinator of abscission (Jacobs, 1955; Addicott *et al.* 1955), but anti-auxins may also be involved (Eaton and Ergle, 1954; Hall and Liverman, 1956; Osborne, 1955). These hypotheses in relation to abscission of flowers in lupins are considered here in the following study of the effects of replacing flowers with lanolin preparations of various auxins, anti-auxins, and growth inhibitors, as well as the effects of various defoliation treatments and substitution of lateral buds with growth-substance preparations.

MATERIALS AND METHODS

The experiments were carried out in the field during the 1955/6, 1956/7, and 1957/8 seasons.

Plants (var. Weiko II) were grown at 1 ft. (30 cm.) spacing in rows 21 in. (53 cm.) apart. Treatments were arranged in a randomized block design with 3–5 replications of 6 plants each, making a total of 18–30 plants included in each treatment.

The defoliation treatments will be described in the text.

The growth substances were applied on the main inflorescence in four different ways:

A. The main inflorescence normally consisted of 8–10 whorls with 5 flowers each. Flowers on the basal three whorls (whorls 1 to 3) were removed and the lanolin preparations were applied on the pedicel marks.

B. As in A, but only two flowers per whorl on one side of the inflorescence were replaced.

C. The top of the inflorescence was cut off just under the whorl 6 or all flower buds were pulled off above the whorl 5 and lanolin was applied either to the cut surface or to the pedicel marks.

The amount of lanolin applied on the pedicel marks was approximately 1 mg. Hence at a 0·1 per cent. concentration 1 μg of growth substance was applied on each pedicel mark. The quantity which actually entered the plant can only be a fraction of this amount and as in other experiments of this kind, cannot be estimated.

Applications on laterals were made either to the top of the lateral after removal of the growing points or as a ring of about 5 mm. wide at the base of the lateral. All treatments were applied on the day that flowers on the lowest whorl of an inflorescence opened.

Flowers and small pods were counted every second day after opening and at longer intervals as pods attained maximum size. The lanolin preparations were prepared immediately before use by dissolving the required amount of substance in a small quantity of absolute alcohol which was then added to the lanolin and thoroughly stirred through until the alcohol had evaporated.

RESULTS

A. *Substitution of flowers by growth substances at the base of the inflorescence*

Expt. 1A (1955/6). The results of this experiment showed that α-naphthyl acetic acid (NAA) was much more active in inducing abscission than β-indolyl acetic acid (IAA); both increased abscission of flowers in the immediate vicinity of the site of application while 2:3:5 triiodobenzoic acid (TIBA) and N-*m*-tolylphthalamic acid (TPA) mainly increased abscission of the flowers

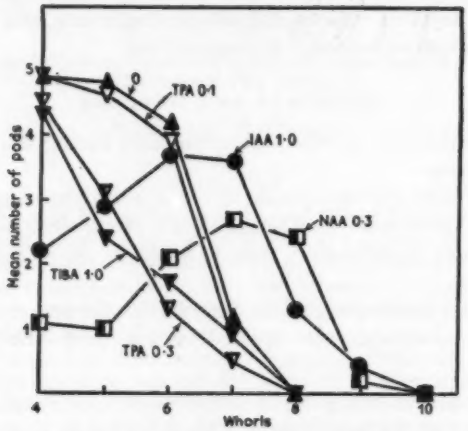

FIG. 1. Numbers of pods setting shown as ordinates on each individual remaining whorl when the basal three whorls are replaced by plain lanolin or by growth substance preparations. Abscissae show remaining whorls numbered 4–10 from base to top.

on the more apical whorls (Fig. 1). These will be termed auxin-types and anti-auxin types of abscission induction respectively. Further results of Expt. 1A are not presented here as they are essentially similar to those of Expt. 2.

Expt. 2 (1956/7) provided more information about possible interactions between NAA, IAA, and TIBA.

The auxin pattern could be clearly distinguished from the anti-auxin pattern of abscission induction (Figs. 2 and 3). Fig. 3 also shows that pod setting on whorls 4 to 7 of the intact control plants is equivalent to that induced by a concentration of TIBA intermediate between 1 and 3 per cent.

When applied in a mixture, IAA acted antagonistically with 0·1 and 0·3 per cent. TIBA on basal whorls except for the mixture of 1 per cent. IAA and 0·1 per cent. TIBA. However, when TIBA was present at 1 per cent. and 3 per cent. in the mixtures, IAA at all concentrations had little effect, except that the auxin type of abscission induction prevailed in those mixtures in which IAA was present at 1 per cent. concentration.

TIBA and NAA acted antagonistically to each other in basal as well as

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Table I

The mean number of pods per whorl (a *and* b) *and the mean total number with S.E. of mean* (*T*) *of pods on plants of which flowers at the base of the inflorescence have been substituted by growth substances. Mean for whorls 4 and 5, column* b (*base*)*; mean for whorls 7 and 8, column a* (*apex*)

Treatments	0			TIBA, 0·1%			TIBA, 0·3%			TIBA, 1·0%			TIBA, 3·0%		
%	b	a	T	b	a	T	b	a	T	b	a	T	b	a	T
0	4·9	1·5	17·0±0·69	3·8	0·8	11·8±0·44	3·3	1·2	10·4±0·41	2·5	0·4	6·1±0·58	0·5	0·1	1·2±0·16
IAA, 0·1	5·0	1·2	16·8±0·51	4·5	1·1	14·6±0·41	3·4	1·2	11·5±0·52	2·1	0·1	4·4±0·24	0·5	0	1·0±0·17
„ 0·3	4·8	1·6	17·5±0·52	4·9	0·9	15·7±0·37	4·5	0·9	12·8±0·57	1·4	0·2	3·4±0·40	0·3	0	0·7±0·13
„ 1·0	2·3	2·1	13·6±0·84	1·4	2·1	9·8±0·88	3·0	0·9	11·5±0·56	1·4	0·3	4·0±0·59	0·3	0·1	0·8±0·17
NAA, 0·1	0·3	3·6	11·1±0·88	2·8	1·6	11·8±0·54	—	—	—	—	2·3	0·4	5·6±0·67	—	—	—	—
„ 0·3	0·1	1·4	5·4±0·89	1·0	1·5	5·9±0·88	—	—	—	—	0·8	0·2	2·3±0·33	—	—	—	—
„ 1·0	0	0	0·2±0·17	0	0	0 —	—	—	—	—	0	0	0·1±0·07	—	—	—	—

apical whorls (Table I). However, in both series of mixtures of 0·1 and 1·0 per cent. TIBA with 0·1 per cent. NAA the effect of TIBA prevailed while in mixtures with 0·3 and 1·0 per cent. NAA the effect of NAA was relatively stronger. Consequently the effect of the mixtures on the total number of pods was about equal to the effects of the most active component by itself.

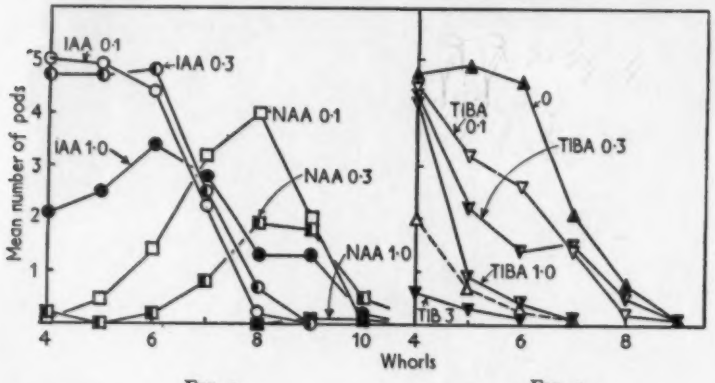

FIG. 2 FIG. 3

FIGS. 2 and 3. Numbers of pods setting on each individual whorl in intact plants (--Δ--) and when the basal three whorls are replaced by various concentrations of IAA, NAA, or TIBA, or plain lanolin (record marked o).

B. *Substitution of flowers by growth substances at one side of the inflorescence*

Expt. 1B. Although all growth-substance preparations were used in this experiment only the results of the most relevant treatments are presented in Table II. Percentages of flower abscission were determined for the side on which flowers were replaced (treatment side with 2 flowers removed per whorl from whorls 1 to 3) and for the other side of the flower stalk (control side from which flowers were not removed).

TABLE II

The percentage of flower abscission on the treated and control side of inflorescences in which 2 flowers per whorl on whorls 1–3 have been substituted by growth-substance preparations (Expt. 1B).

(*a*) Treated side of the flower stalk (2 flowers per whorl, except for whorls 1–3).
(*b*) Control side of the flower stalk (3 flowers per whorl).

Treatments %	Mean of whorls 1–3		Mean of whorls 4–5		Mean of whorls 6–7		Mean of whorls 8–9	
	a	b	a	b	a	b	a	b
Plain lanolin . .	—	3	17	54	80	86	100	100
IAA 1·0 . .	—	4	50	44	61	97	98	100
NAA 0·1 . .	—	4	41	53	68	98	100	100
,, 0·3 . .	—	23	94	86	83	91	91	97
,, 1·0 . .	—	35	100	92	100	99	100	100
TIBA 10·0 . .	—	10	100	52	100	98	100	100

The abscission figures of whorls 4 and 5 of the control treatment show that the abscission-inducing influence of pods on the basal three whorls was in strictly vertical direction. When IAA and NAA were applied at the 1·0 and 0·1 per cent. concentration respectively then the same vertical effect was obtained. However, with 0·3 and 1·0 per cent. NAA, flower abscission on the control side of the flower stalk became quite pronounced from the second whorl onwards, showing that at high concentrations the effect of NAA could move in lateral direction. TIBA even at a very high concentration acted in strictly vertical direction also on the more apical part of the flower stalk.

C. *Substitution of flowers at the apical part of the inflorescence*

Expt. 1C. Growth substances were either applied to the cut surface of the flower stalk above whorl 5 or to the pedicel marks of the removed flower buds. The results of both application techniques were identical and were therefore taken together (Table III).

TABLE III

The mean number of pods per whorl and the mean total number of pods with S.E. of mean on plants of which flower buds at the apical part of the inflorescence have been substituted by growth substances. Oa is a control with plain lanolin; Ob is a control in which the inflorescence was left intact (Expt. 1C). (CPA is p-chlorophenoxyacetic acid)

Treatments %	Mean number of pods on whorls 1–3	4	5	Total pods	Treatments %	Mean number of pods on whorls 1–3	4	5	Total pods
Oa	4·6	2·9	0·5	17·4±0·63	CPA 0·1	4·8	2·9	0·9	18·4±0·67
Ob	4·7	2·9	0·7	16·8±0·62	,, 1·0	4·6	4·1	0·5	18·4±1·05
IAA, 0·1	4·9	3·9	1·3	19·7±0·67	TIBA 0·1	4·7	2·6	0·7	17·3±0·73
,, 0·3	4·7	3·0	0·9	18·1±0·71	,, 1·0	4·8	3·1	0·5	18·1±0·50
,, 1·0	4·7	2·8	0·5	17·6±0·54	,, 10·0	4·9	4·1	0·1	18·8±0·42
NAA, 0·1	4·6	1·4	0·5	15·7±0·64	TPA, 0·1	4·8	3·1	0·8	18·5±0·67
,, 0·3	4·6	1·6	0·4	15·8±0·89	,, 0·3	4·8	2·7	1·1	18·3±0·77
,, 1·0	4·7	0·9	0	15·1±0·34	,, 1·0	4·1	0·4	0	12·7±0·71

They show that in basipetal direction none of the growth substances had much effect on flower abscission. At high concentrations NAA and TPA prevented pod setting on whorl 5 only and reduced pod setting on whorl 4.

It was rather surprising that the effect was not any greater as flowers on whorl 5 were only a few millimetres from the site of application when plants were treated; IAA especially was expected to be transported better towards the base of the flower stalk. Although with several substances the total number of pods was slightly increased with apical application, none of the differences were significant except for the 0·1 per cent. IAA treatment. The difference, however, was small and unless repeated it would be premature to attach any physiological significance to this result.

D. *Effects of defoliation and removal of lateral growth*

Expt. 3 (1956/7). Continuous observation on abscission of flowers enabled a clear-cut distinction to be made between flower abscission without apparent growth of ovaries, and pod abscission of small pods at least 15 mm. long.

Flower abscission. Fig. 4 shows that control plants (treatment 1) dropped most of their flowers before ovaries started to grow.

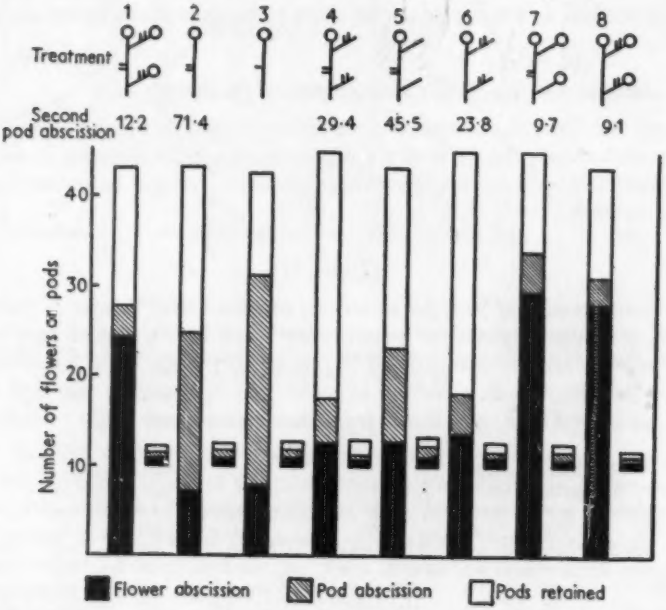

FIG. 4. The effect of removal of whole laterals, lateral buds, and partial defoliation on flower- and pod-abscission. The treatment symbols show main stem as a vertical line, laterals sloping, leaves as short vertical or horizontal lines, growing points as circles.

The figures immediately below the treatment symbols give the amount of pod abscission in the second period as a percentage of the total abscission.

When all laterals of the plant were removed (treatment 2) only a few flowers of the apical whorls dropped without apparent growth of ovaries, and a substantial number dropped as small pods. The delay in abscission occurred in spite of a considerable reduction in total leaf-area on the plants (Table IV). When the leaf area was further reduced by halving the number of leaflets on the main stem, flower abscission was almost unchanged (Fig. 4, treatment 3).

The effects of defoliation on flower abscission depended to some extent on the presence of terminal and axillary buds on the laterals. When they were absent, defoliation of all laterals or of the main axis had practically no effect on flower abscission (Fig. 4, treatments 4, 5, and 6).

TABLE IV

Fresh weights (g.) and leaf areas (cm.²) with S.E. of means of various parts of lupin plants on the day of treatment. (Average of 7 random plants.)

	Main axis	Laterals	Total plants
Leaves f.w. . . .	19·9±0·77	49·0±2·40	68·9±2·38
Leaves area . . .	366·4±14·3	1,057·0±51·7	1,423·4±48·4
Petioles f.w. . . .	10·0±0·50	24·4±1·49	34·4±1·58
Stems f.w.	12·6±0·96	37·8±3·06	50·4±3·28
Infl. buds f.w. . . .	3·4±0·23	8·0±0·74	11·4±0·68
Number of laterals (average of 18 plants) . . .	—	8·0±0·23	—

When the lateral growing points were present lateral defoliation had a significant effect on abscission (Fig. 4, treatments 1, 7, and 8).

In treatment 4 only the terminal and axillary buds were removed from all laterals. Again, flower abscission was substantially less than on normal plants (Fig. 4), and although abscission was significantly higher than on plants from which the laterals were entirely removed, it can be inferred that most of the stimulation of flower abscission was due to the presence of growing tips on the laterals, and that the balance was due to the presence of lateral stems and developing pods on the main stem.

Pod abscission. When pod abscission is expressed as a percentage of total abscission it becomes evident that the abscission of pods was relatively unimportant in each of those treatments in which the growing points were left intact (Fig. 4, treatments 1, 7, and 8). On removal of the growing points pod abscission was always relatively important (treatments 2, 4, 5, and 6). Although in each of the latter treatments a high number of ovaries was induced to grow it appeared that generally the plant could not support all these to maturity.

Plants with an undisturbed leaf area (treatment 4) retained 84 per cent. of their growing ovaries while plants with their entire laterals removed (treatment 2) retained 52 per cent. and plants with only half the leaves on their main stem (treatment 3) retained only 38 per cent. of their growing ovaries. The mean number of mature pods for these treatments was 27·5, 18·9, and 13·1 respectively.

These results suggest that pod abscission depends on the extent of the preceding flower abscission and also on the number of mature pods a plant can support without overtaxing its assimilative capacity.

The final number of pods present on the main axis for treatments 7 and 8 was 11·5 and 12·0. This shows that it made little difference whether the required nutrients had to be translocated from leaves on the main stem or from leaves on laterals.

E. *Application of growth substances on laterals*

Expt. 4 (1956/7). As it was found in the previous experiment that the presence of growing points on laterals strongly contributed to the abscission

TABLE V

Flower abscission on plants in which lateral buds were substituted by plain lanolin (treatments L1, L2), 1 per cent. NAA (N1, N2) or 1 per cent. TIBA (T1, T2). 1 per cent. NAA and 1 per cent. TIBA were applied at the base of laterals in treatments N3, N4, and T3, T4 respectively. Main stems were defoliated in treatments L2, T2, T4, N2, and N4

Treatments	Percentage of flowers dropped in whorls 1–3	4–6	7–9	Mean total number of flowers dropped and S.E. of mean
Control	0·3	39·0	98·8	17·9±0·69
L1				3·3±0·94
L2	0·3	4·1	38·7	4·6±0·84
N1				4·3±0·39
N2				9·8±1·18
N3				4·8±0·96
N4	81·0	19·7	33·0	18·9±1·19
T1				2·8±0·67
T2				5·8±0·54
T3				3·4±0·84
T4	0·3	4·3	48·8	7·6±0·89

of flowers, all lateral growing points were removed and substituted by 1 to 8 mg. of the growth substance preparations.

IAA, TIBA, and *p*-chlorophenoxyacetic acid (CPA) did not have any significant effect on flower abscission at all concentrations tested. Only 0·3 and 1·0 per cent. NAA induced a significant but much smaller increase than that caused by the presence of lateral buds. (Mean flower abscission from plants with lateral buds, controls with plain lanolin, 0·3 per cent. NAA, and 1·0 per cent. NAA being 27·1, 7·2, 10·8, and 12·9 respectively.)

Expt. 5 (1957/8). Apparently the applied growth substances were not readily transported from the laterals to the main flower stalk even though sites of application on the apical laterals were only 2–3 cm. removed from the base of the flower stalk. As auxin transport has been found to depend on the photosynthetic production of carbohydrates (von Guttenberg and Zetschke, 1956) and several other reports have been made that 2:4-dichlorophenoxyacetic acid translocation is dependent on the carbohydrate supply of the treated leaves (Rohrbaugh and Rice, 1949; Mitchell and Brown, 1946; Weaver and De Rose, 1946), it appears that these growth substances move together with the carbohydrate stream. In Expt. 3 it was shown that nutrients required for pod growth could be drawn equally well from leaves on the main stem as from leaves on laterals (cf. treatments 7 and 8). Main stems, therefore, were defoliated in an attempt to induce the carbohydrate stream to run from the laterals to the main inflorescence. The results in Table V indeed show that the growth substances (NAA especially) became much more effective when they were applied in combination with the defoliation of the main stem (treatments N_2 and T_2); this effect became even more pronounced when the substances were applied to the base of the laterals (treatments N_4 and T_4).

Again NAA-induced abscission occurred predominantly on the basal whorls while TIBA-induced abscission occurred on the apical whorls, the latter being more consistent with the normal pattern of abscission (cf. Table V).

DISCUSSION

Auxins (IAA, NAA) as well as anti-auxins (TIBA, TPA) can induce flower abscission when applied at the base of the lupin inflorescence. However, the pattern of abscission induced with auxins differed from that induced with anti-auxins and only the latter type was similar to the pattern of abscission normally caused by developing pods on basal whorls, i.e. the abscission-inducing effect of the pods on the first three whorls could be imitated by replacing them with TIBA on these whorls. Considering this evidence, it appears that an anti-auxin or inhibitor rather than auxin dominates in the induction of flower abscission in lupins. Recently evidence has been presented that very young cotton bolls produce a substance which inhibits auxin activity (Carns *et al.*, 1955), and also the fluctuating amounts of auxins and the increasing amount of inhibitors produced during flower and fruit development in apples (Luckwill, 1957) and black currants (Wright, 1956) provide further support for the above hypothesis.

It is very unlikely that the above results were due to toxic effects. TIBA, for instance, even caused the internodes between the whorls on which it was applied to elongate more than in the controls (Van Steveninck, unpublished results). The only evidence of toxic effects was obtained with γ-phenylbutyric acid which at 10 per cent. concentration caused flowers to shrivel and to remain on the flower stalks for several weeks until pods on other plants had matured.

Our experiments with mixtures of TIBA, NAA, and IAA showed that these substances acted antagonistically on abscission provided one considered basal and apical whorls separately. The antagonistic effect, however, was far less pronounced than shown by Weintraub *et al.* (1952) in beans.

The difference in abscission-inducing activity of IAA and NAA could have been due to enzymatic destruction of IAA on the surface of damaged cells and subsequently in the stem tissue of the flower stalk; or transport of IAA in acropetal direction may not have been as efficient as with NAA. This complies with the inefficient translocation and possible enzymatic destruction of IAA in lupin tissue mentioned by Söding (1951).

The relatively small effects of growth substances applied to only one side of the inflorescence on flowers at the other side suggest that they are not readily transported in lateral direction. This probably explains the previous finding that pod setting on basal whorls is nearly always complete and pods *within* whorls do not seem to have an abscission inducing effect on each other (Van Steveninck, 1957).

Especially, in the experiments in which laterals or terminal and axillary buds were removed from the plant, it became quite noticeable that two different types of abscission could be distinguished, viz. abscission of flowers

without apparent growth of ovaries and abscission of small pods of at least 15 mm. long. Flower abscission is the rule on normal plants, and it seems to be mainly caused by the presence of laterals which always grow vigorously at the time that the main stem flowers. Removal of these laterals or checking their growth by removing their apical buds delayed abscission, and nearly all flowers produced small pods except for those on the uppermost one or two whorls of the main inflorescence. This residual flower abscission on plants with no lateral growth was probably due to the developing pods on the main stem (Van Steveninck, 1957) and was not affected by radical changes of leaf area (cf. Expt. 3, Table IV and Fig. 4). On the other hand, the abscission of small pods was found to depend on leaf area suggesting that pod abscission is activated when the assimilative capacity of the plant becomes insufficient to maintain all the pods.

Although the same growth substances were quite active in inducing abscission when applied at the base of the inflorescence they had no, or only a slight effect on flower abscission when applied on the laterals. However, in a further experiment in which main stems were defoliated and applications were made at the base of the laterals the effect of the growth substances became much stronger, indicating that applied growth substances are not readily transported from the laterals to the main flower stalk unless the carbohydrate stream is induced to run in this direction by defoliating the main stem. This conforms very well with the observations made by von Guttenberg and Zetschke (1956) that auxin transport depends on the photosynthetic production of carbohydrates.

Again the NAA-induced abscission was atypical while the TIBA-induced abscission approached the normal pattern of abscission.

Although it was not possible to explain the correlation effect of lateral growth upon flower abscission by using known auxins or anti-auxins, the fact that flower abscission could not be directly related to a shortage of assimilates in the plant, indicates that some other abscission-inducing substance, which can move freely through the plant remains to be discovered.

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Observations on the Gaseous Exchanges which take place between *Menyanthes Trifoliata* L. and its Environment

Part II

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SUMMARY

The exchange of gases between whole plants of *Menyanthes* and their environment has been investigated. When a 45 cm. rhizome is surrounded by an almost oxygen-free environment, leaving the leafy shoot exposed to air, some oxygen passes right through the rhizome, and the Q_{O_2} of the plant is significantly reduced.

Analyses of gases from the air-space system show that oxygen concentrations fall and CO_2 concentrations rise when plants are transferred to, and maintained under conditions like these. Oxygen concentrations show a linear gradient from top to bottom of the plant, whilst the reverse is true for CO_2.

The main barrier to gas movement is shown to lie within the air-space system of the rhizome, and the number of leaves present on the plant has little effect upon the oxygen content of the internal atmosphere.

It is shown that as the concentration of CO_2 increases in the air space system of a rhizome, so the consumption of oxygen falls off, thus allowing more oxygen to pass through the rhizome to more distal regions.

Variation in the seasonal activity of the plant is discussed in relation to these findings and to reported studies on the respiration of rhizome-tissue slices. Determinations of respiration on tissues such as these are held to be less meaningful if carried out according to the 'direct' method.

The biological significance of these features is discussed.

Introduction

In the first paper of this series (1951) the authors gave an account of some preliminary analyses of the internal gases of the rhizome and roots of *Menyanthes trifoliata* L. The present communication describes the outcome of further work on the gaseous exchanges between *Menyanthes* and its environment; it has led to a modification and to a considerable extension of the views then expressed.[1]

Methods and Apparatus

The form of the apparatus used to determine gas exchange may be seen in Fig. 1. It consisted essentially of an upper and a lower chamber, separated

[1] The untimely death in June 1953 of Kenneth Vallance has necessitated considerable delay in publication; in its present form the paper mostly represents our joint work and discussions, though the final editing, their presentation, and the opinions expressed must now be my whole responsibility. It is fitting that I should here pay tribute to an alliance which meant a very great deal to both of us.—D. A. C.

from one another by a wax seal which was traversed by the stem of the plant used in each experiment. Most versions of the apparatus that were used in the later experiments differed from the illustration only in terms of the respective volumes of upper and lower chambers. Generally the upper chamber had a volume of about 350 ml. and the lower chamber was larger by about 40 ml. The luting wax consisted of a 1:1 mixture of beeswax and paraffin wax, and proved quite satisfactory in use.

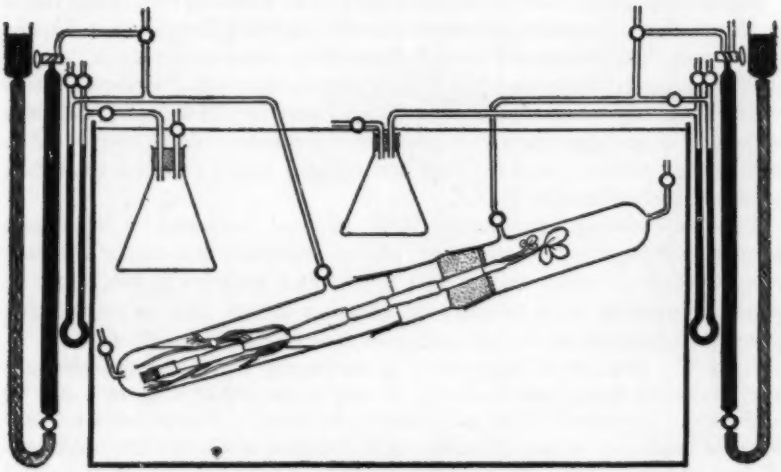

FIG. 1. Gas-exchange apparatus (for details see text).

The plant, generally about 45 cm. long, was sealed into the apparatus in such a way that only the leafy shoot emerged from the seal into the upper chamber, whilst the lower chamber was occupied by the green rhizome and its attached root system together with a small known volume of dilute culture solution. The distal end of the rhizome was sealed off with a mercury seal, so that all diffusion into or out of the lower chamber took place over the surface of the rhizome and its attached roots.

The apparatus may be thought of as a modified large-scale dual Barcroft respirometer of the constant-pressure type, with a side-arm and mercury burette whereby volume changes may be measured directly. Similar principles were employed here (for details, see Dixon (1951), p. 6). The required gas mixtures were flushed through top and bottom chambers by controlling the inlet and outlet taps provided. By moving the mercury up and down in the gas burettes by means of the mercury reservoirs, it was possible to ensure thorough mixing of the chosen gas through all parts of each system, top and bottom. Samples were taken from both chambers at the beginning and end of each experimental period, and were subsequently analysed in a Haldane

apparatus. The total volumes of gas in both chambers could be measured to the nearest 0·05 ml. before and after each experiment.

After making a volume correction for the gases dissolved in the known volume of fluid in the lower chamber, it was possible to calculate the gain or loss of CO_2 and oxygen by each system, and thus to estimate both the gas exchange of the plant and the movement of gases from one chamber to another through the rhizome. Plant and apparatus were kept in the dark in a constant-temperature bath at 22° C.

It should be made clear at this point that the structure of the ground tissue in *Menyanthes* is such that gas movement can take place through the air-space system only by diffusion, and never by mass flow, however restricted. Because of this, pressure differences could be built up and maintained between the two chambers during an experiment; by raising or lowering the mercury reservoirs at the end of an experiment such pressure differences could be levelled off to atmospheric pressure, and the resultant changes in gas volumes were then measured and reduced to N.T.P.

The gas-exchange experiments were designed primarily to investigate oxygen movement within the plant, and to determine the extent to which oxygen enters the plant via the leafy shoot; as a corollary it was hoped to obtain information as to its entry by accessory means, such as by diffusion across the epidermis of rhizome and roots under suitable conditions.

Since the rhizome of *Menyanthes* is commonly almost completely submerged, under conditions in which the oxygen content of the water may be negligible, a first series of gas-exchange experiments was undertaken to compare the behaviour of individual plants in darkness when subjected consecutively to three sets of conditions. These treatments are conveniently described in abbreviated form as: Air Top/Air Bottom (or T_1) and Air Top/Nitrogen Bottom (or T_2) and Air Top/Nitrogen Bottom/Shoot removed (or T_3). It was believed that, under the last set of conditions, the removal of the leafy shoot[1] might provide evidence as to whether resistance to the entry of oxygen into the plant by diffusion was in any way localized in the leaves.

In three out of the five experimental runs from the results of which means have been extracted for Fig. 2, the following was the sequence of operations. Gas exchange was determined in the usual way for the whole of a 45 cm. plant during the first 24 hours in the dark. The plant was then exposed in light of low intensity to Air Top/Nitrogen Bottom conditions and allowed to equilibrate during the next 24 hours. On the third day these conditions were repeated, but with the plant now in the dark. At the beginning of the fourth day the shoot was removed, and gas exchange was measured over this next 24-hour period in the dark under Air Top/Nitrogen Bottom/Shoot removed conditions. Meanwhile the detached shoot after brief washing was placed in

[1] Here, as on all succeeding occasions mentioned in this paper, estimates of rhizome length, together with all sampling levels, were measured from the level of insertion of the lowest functioning leaf. 'Decapitation' refers to the removal of the leafy shoot by a transverse cut at this level.

a closed respirometer system essentially equivalent to the top chamber of the gas-exchange apparatus and its rate of oxygen intake and CO_2 output were determined at 22° C. in air. During the fifth day the decapitated stem was split longitudinally, and the two halves were then well washed for an hour in running water; their CO_2 output was determined at a relatively low oxygen

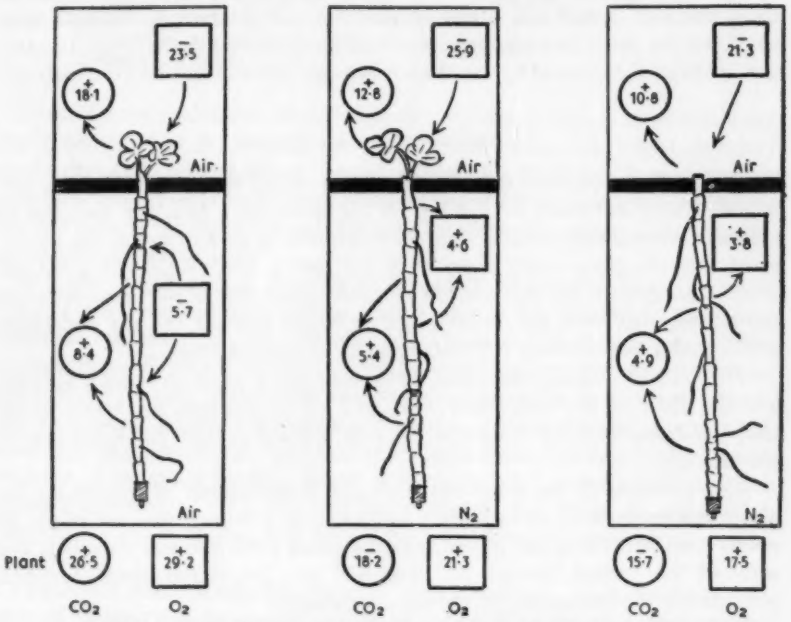

FIG. 2. Diagram to show comparisons of gas exchanges in plants of *Menyanthes* under Air/Air and Air/Nitrogen conditions. Means for five plants expressed as μl. gas/g. fresh wt./hour.

5% Confidence Intervals for differences between plant means, calculated according to Tukey's test (Snedecor (1956)): $D_{O_2} = 7{\cdot}7$ and $D_{CO_2} = 9{\cdot}1$.

tension, this time by the Pettenkoffer method with a gas stream of 7–9 per cent. of oxygen; it is unusual to encounter concentrations of oxygen much below this level in the stems of Air Top/Nitrogen Bottom plants.

In a second series of respiratory-chamber experiments an attempt was made to find out how much oxygen could be taken up under highly favourable conditions by the rhizome itself. In this series, Air Top/Air Bottom (T_1) conditions were compared on the first day with Nitrogen Top/Air Bottom (T_4) conditions on the second day. On the third day the Nitrogen Top/Air Bottom conditions were repeated, but a potassium cyanide solution was added to the culture solution in the lower chamber to give a final concentration of 10^{-3} M. KCN (T_5). The use of nitrogen in the upper chamber was intended to accentuate the diffusion gradient of oxygen between the two chambers. It

was argued furthermore that if oxygen movement into and within the plant were controlled in part by metabolic means, for example, at the surface of the root, or during phloem conduction perhaps, one would expect by such a poisoning experiment to be able to reduce this contribution to the total oxygen uptake, thereby enabling more oxygen to diffuse through to the top chamber.

A series of general investigations into the respiratory behaviour of tissue slices was also carried out. Standard Barcroft and Warburg techniques were employed for these investigations, and a comparison was made of the respiration of slices as measured by the 'direct' and the 'indirect' method of Warburg.

Experimental Results

Data from gas chamber experiments. Fig. 2 shows some of the results obtained. They are based on the figures for five plants and, for the sake of convenience and comparison, they are expressed as mean rates of gas movement, and are given as μl. of gas at N.T.P. per g. fresh weight of plant per hour. Changes in the gas content of both upper and lower chambers are shown, together with the derived figures which indicate the gas exchanges which took place between the plants and their surroundings.

Where air surrounded the plant, about four times as much oxygen diffused into the plant via the leafy shoot as entered via the rhizome and roots, whilst CO_2 loss from the leafy shoot was just over twice that from the lower part of the plant.

With nitrogen in the lower chamber, the consumption of oxygen by the plant was reduced by one-quarter, while the CO_2 output was cut down by nearly one-third. Oxygen passed into the plant from the top chamber, but some of this passed through the plant and was lost to the lower chamber, presumably via the surface of the rhizome system.

The values for oxygen uptake and CO_2 output in the five plants under treatments T_1, T_2, and T_3 were subjected to analysis of variance. The analysis confirmed that both treatments involving Nitrogen Bottom conditions had brought about a highly significant lowering of the overall respiration-rate in comparison with plants under Air Bottom conditions (T_1). This enabled more oxygen to diffuse downwards through the plant and out across its lower surfaces and to appear in the lower chamber. So far as oxygen uptake was concerned, the difference between decapitated and whole plants under Nitrogen Bottom conditions was significant at a probability level lying between 5 and 10 per cent. If this is, even with reserve, to be taken as representing a real difference, it may best be explained as resulting from the removal of the metabolically more active shoot apex from the total of the respiring tissues present. So far as CO_2 output is concerned, the difference between plants under treatments T_2 and T_3 is not significant.

In other words, under these experimental conditions, decapitation of the stem is without much effect on the gas exchange of the rhizome. It seems most unlikely that the leaves are operating as the site of a resistance to diffusion of

any magnitude by comparison with the resistance offered by the remainder of the diffusion path.

The remaining data from the first series of chamber experiments are briefly summarized below. Figures as before represent μl. gas/g. fresh wt./hr.

	O_2 uptake	CO_2 output
Detached shoot apex respired in air . .	66±11	64±8
Decapitated stem, split longitudinally and respired in 7–8 per cent. oxygen stream in Pettenkoffer tubes.		55±6

These figures enable one to calculate that the shoot apex is responsible for nearly one-third of the oxygen absorbed by the whole plant under chamber conditions; they further show that the availability of oxygen is increased at most two- to three-fold by longitudinal splitting of the stem, whereas decapitation has little or no effect beyond that resulting from the removal of the metabolically more active apex.

By splitting such a stem, the effective length of the diffusion path is reduced from 45 cm. longitudinally to less than 1 cm. in the transverse direction, whilst the rate of oxygen consumption only increases two or three times. This suggests that diffusion is easier in a longitudinal than in a transverse direction, and support is given to this view on anatomical grounds. In any case, tissue-slice data show, under comparable conditions, optimal rates of oxygen consumption which are about twice that of the split stem. Not only is the inward diffusion of oxygen a limiting factor in these chamber experiments, but the outward diffusion of CO_2 also will be hampered, and it can be anticipated that both of these factors will have their repercussions upon oxygen consumption.

Tissue-slice data. Using the Barcroft direct method, an assessment was made of the decrease in respiration-rate of 1 mm. slices sampled from successively lower levels down a 45-cm. stem and washed overnight. The gradient in respiration-rate was shown to be significantly linear, but was nevertheless irregular; this is hardly surprising if one remembers that local meristematic activity (in bud or adventitious root production) may introduce appreciable sources of variation. The mean Q_{O_2} showed a gradient from 215±14 for the 0 to 7·5 cm. zone down to 177±14 for the 37·5 to 45 cm. zone, a decrease of about 20 per cent.

This relatively high level was never again detected in tissue slices taken at other times of the year, and suggests the probability that there is a seasonal variation in the respiration-rate of *Menyanthes*. The figures quoted were obtained in May, at a time of active leaf expansion. Comparable figures for September indicate values for Q_{O_2} of the order of 150–60 after prolonged washing, and less than half of this value after one hour of washing.

Further data were obtained in September 1953 using the Warburg respirometer with 1·5 mm. slices, washed either for 1 or 40 hours. At the same time a comparison was made between the indirect and direct methods (see

Umbreit *et al.* (1949)). All tissues were sampled from the 15 to 22·5 cm. region, and the results are tabulated below.

Method	Washing period (hours)	O_2 uptake	CO_2 output
Direct	·1	61±2	71±3
Indirect	1	34±2	39±2
Direct	40	156±5	181±7
Indirect	40	128±5	142±6

The complete removal of CO_2, in the presence of the caustic potash employed in the direct method, caused a highly significant increase in respiration; the Q_{O_2} for tissues washed for 40 hours increased by 20 per cent., for those washed for 1 hour only by 80 per cent. It is clear that the use of the direct method is inadmissible in a tissue of this nature. The increased respiration-rate due to prolonged washing will be discussed more fully at the end of this paper.

Uptake of oxygen at the rhizome surface. The uptake of oxygen from a bottom chamber containing air was of some interest, for it provided, under highly favourable conditions, some measure of the capacity for entry of oxygen into the plant by routes other than via the leaves. It is likely that there are periods in which dissolved oxygen may be available to the plant by diffusion into the submerged rhizome; indeed there are times during winter flooding when, apart from the oxygen released by photosynthesis within the green tissues of the underwater rhizome, supply by diffusion from the surrounding water will be the only available source.

In this second series of gas-exchange experiments, plants were subjected on three consecutive days, firstly to conditions in which oxygen was available from both top and bottom chambers (treatment T_1 as before), then to conditions in which the oxygen gradient from below was steepened by replacing the air of the top chamber with cylinder nitrogen (treatment T_4). On the third day, whilst repeating these gas conditions, potassium cyanide solution was added to the fluid in the lower chamber to give a final concentration of M/1000 (treatment T_5). Three replicate experiments were performed.

Fig. 3 shows the results obtained. The values for intake of oxygen, expressed as a mean for three plants (in μl. O_2/g. fresh wt./hour) show that when oxygen was equally available to both leafy shoot and rhizome surface, the intake from below was as low as 20 per cent. of the total. About twice as much CO_2 was lost through the leafy shoot into the upper chamber as was lost by diffusion from roots and rhizome into the lower chamber.

With nitrogen filling the upper chamber, the total oxygen intake was cut down to about a fifth of its value in total air, and a little oxygen was actually lost to the top chamber. The uptake of oxygen from the bottom chamber was very much the same under both treatments. Under these Nitrogen Top/Air Bottom conditions the overall CO_2 output was reduced to just under three-quarters of its value in air.

The effect of adding KCN to the small amount of fluid in the lower chamber

(under Nitrogen Top/Air Bottom conditions) was not very marked. Oxygen intake by the plant was reduced a little further, but analysis of variance showed that treatment T_4 did not differ significantly in this respect from treatment T_5. However, the total CO_2 output of the plant was significantly reduced by one-third under KCN treatment. Of the CO_2 produced, a large proportion

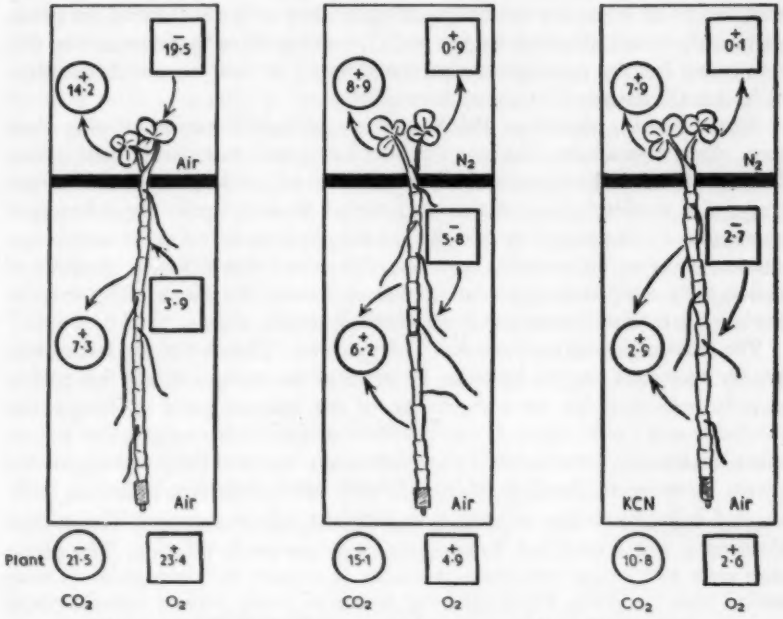

FIG. 3. Diagram to show movement of gases across the surface of roots and rhizomes of *Menyanthes*. Means of three replicates, expressed in μl. gas/g. fresh wt./hour. 5% Confidence Intervals (Tukey's test) for plant means:

$$D_{O_2} = 6{\cdot}8 \text{ and } D_{CO_2} = 2{\cdot}6$$

appeared to be released into the upper chamber and less into the lower chamber. It is possible that this can best be explained in terms of differences in the intensity of poisoning of basal and apical portions of the plant under these circumstances.

The amount of oxygen which diffused through into the top chamber was very small and did not differ notably from that passing out from unpoisoned plants under comparable conditions. It could be argued that the reduction by one-half in the uptake of oxygen by the plant after poisoning was due either to the suppression of metabolic activity controlling uptake at the surface of the plant, most likely in the roots, or to the reduction in total consumption of oxygen by the plant as a whole. However restricted and localized the tissues in which the KCN could have been effective here, its presence must essentially

have led to a diminution in the total metabolic consumption of oxygen. That being so, if physical processes of diffusion only were involved in the movement of oxygen, then one would have expected to find an increased movement into the upper chamber, since less would have been consumed *en route*. In point of fact, less oxygen was observed to pass out from the poisoned plants into the upper chamber, and this would support the first hypothesis, namely that the KCN is in part controlling oxygen entry at the surface of the plant. As already stated, the total output of CO_2 was significantly decreased by this treatment, but the experiment was insufficiently critical to establish statistically that the oxygen differences were valid.

Whilst there is, therefore, this hint that vital uptake may be playing some part, albeit a small one, the point cannot be regarded as clearly established. It must therefore be presumed, in the absence of good evidence to the contrary, that the larger part of the oxygen that is taken up by the submerged rhizome and roots enters by straightforward physical diffusion across the epidermis. It is to be noticed, however, that when the diffusion gradient is deliberately steepened by replacing the air around the shoot with nitrogen, the oxygen intake from below is very little changed.

The internal gaseous environment of Menyanthes. The data of the last section are by no means easy to interpret in terms of the comparatively few results already published for the composition of the internal gases of *Menyanthes* (Vallance and Coult, 1951). It was therefore decided to investigate this feature more intensively. Comparisons were first made between the internal gases of plants growing in darkness at 22° C. with their rhizomes either in well-aerated culture solution or in oxygen-deficient culture solution. The culture fluid used was a modified Knop solution at one-tenth dilution. The plants that were to be kept with their rhizomes in oxygen-deficient medium were sealed into 75 × 5 cm. Pyrex tubes by means of firmly packed non-absorbent cotton wool and a luting wax made of equal parts of vaseline and beeswax. The leafy shoot alone emerged from the seal at the top of the tube, which was filled with freshly boiled and quickly cooled culture solution. Aerated plants were loosely held in similar tubes without a seal, but the surrounding solution was constantly disturbed by an airstream from a compressor.

One-leaved plants, about 50 cm. in length, were subjected to experimental treatment for 2 days, a period which had proved adequate for the attainment of steady conditions in the internal gaseous environment. At the end of this 2 days of treatment, a 22·5–30 cm. portion of rhizome was sampled. Gas analyses, following extractions, were carried out according to the technique described in the previous paper (Vallance and Coult, 1951).

Most of the determinations made after 1951 included, for any selected level, a gas analysis of samples from cortical and stelar tissues separately, in addition to root tissue from this level if available. For the extraction of samples of gas from these two zones in a 7·5 cm. portion of stem, the following method was found to work satisfactorily. Working under water in a deep sink, a length of stem was cut from the rhizome at the chosen level and then divided into three

segments each about 2·5 cm. in length. With a sharp cork-borer of appropriate dimensions, and, for preference, cutting with it into the stem segment from each end, the stele could be removed with a very small amount of adherent cortical tissue. This cortical tissue was then gently scraped in order to expel the small bubbles of cortical air still present. The three segments similarly treated were secured with thin wire (still under water) and rapidly transferred to the extractor. Here they were immediately immersed under the mercury in the trough and manipulated to expel any trapped air-bubbles; they were then introduced into the limb of the extractor and their gases extracted. Samples of from 25 to 50 μl. could be obtained from the stele in this manner, and could be handled satisfactorily in the gas analyser. The remaining cortical segments, stored meantime under water, were then threaded on to wire and again introduced into the extractor. The whole sequence of events could be accomplished with practice within the limits of from 5 to 10 minutes, and it seems reasonable to suppose that little change could have taken place during this period (either by the extraction of gases from solution or by metabolic changes) which would have very markedly affected the internal atmospheres of these segments. Table I and Fig. 4 show the results from experiments which were carried out in two consecutive years.

In 1951 (Table I) determinations were made on six pairs of plants during

TABLE I

A comparison of internal gas concentrations of Menyanthes *plants held under aerated and non-aerated conditions. Means from six replicates.*

T_1 = *Air Top/Aerated culture soln. Bottom*; T_2 = *Air Top/Boiled culture soln. Bottom*

Sampling zone	CO_2		Oxygen	
	T_1	T_2	T_1	T_2
Middle stem . . .	2·8	4·3	14·3	10·9
Middle root . . .	3·1	5·1	14·1	11·2
Treatment means . .	3·0	4·7	14·2	11·1
Least significant differences (at 5% level):				
Overall treatment means . .	1·0		2·6	
Treatments in *one* zone . .	1·3		3·3	

a period of 1 week towards the end of August. In the stem, oxygen was found to be present at a level which was significantly higher in the aerated plants than in the plants which were immersed in oxygen-free culture solution. At the same time, the concentration of CO_2 was significantly higher in both stem and roots of plants maintained in the non-aerated solutions.

The 1952 figures (see Fig. 4) are based upon determinations which involved eight pairs of plants; here the sampling period was earlier and extended over 2 months (mid-May to mid-July). In these determinations cortical and stelar gases were extracted and analysed separately in addition to those gases extracted from the roots. Whilst in this series of determinations the figures show trends comparable with those of the 1951 series, only the figures for

CO_2 levels are acceptable as showing a significant difference between the effects of the two conditions, and it is thought that the essential difference between these and the figures for 1951 may well be a seasonal one. Considering the zonal means for the two treatments, it may be shown by an analysis of

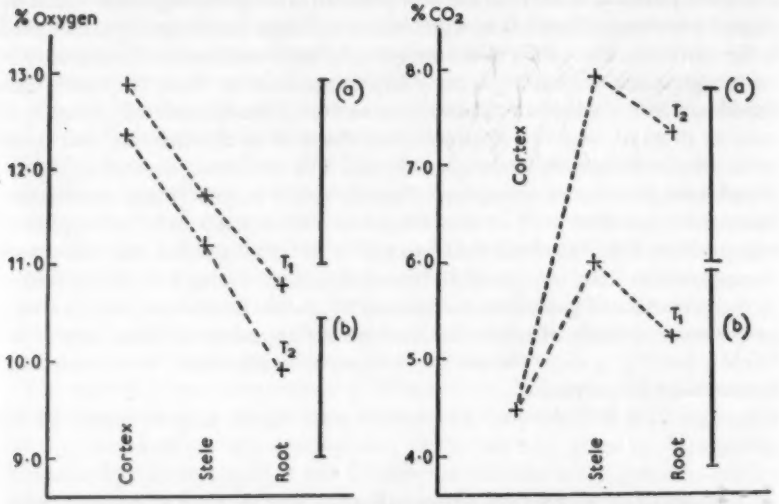

FIG. 4. Comparison of internal atmospheres of plants under treatments involving aerated and air-free conditions. Means of eight plants each sampled at the 30–37·5 cm. level. T_1 = Air Top/Aerated Culture Solution Bottom. T_2 = Air Top/Boiled Culture Solution Bottom.

Least significant differences (5% level) are shown between:
(*a*) treatments in any one zone, and (*b*) overall treatment means.

variance that a zonal gradient in oxygen existed from cortex via stele to root; this was essentially linear and at a high level of significance. It might reasonably have been expected on the anatomical evidence.

On the other hand the mean concentration levels of CO_2 (derived by averaging both treatments) showed significant differences between all zones ($P < 0{\cdot}05$), but stelar CO_2 was higher than that in either cortex or root, which suggests that the diffusion of CO_2 away from the stele is less easy than in the case of either cortex or root. Here again this might have been anticipated on anatomical and positional grounds.

In terms of individual treatments, the differences in CO_2 levels between cortex, stele, and root fail to show significance in the T_1 plants, but are clearly significant in plants under T_2 conditions.

The composition of the internal gases of plants subjected to gas exchange chamber conditions. In an experiment (September 1951), plants about 50 cm. in length were sealed into long Pyrex tubes, held nearly horizontal, containing only just enough culture solution to cover the roots. Cylinder nitrogen or laboratory air was blown through each tube for 1 hour and the tubes were

then closed off, and left for 48 hours in the dark at 22° C. Analyses were then carried out on the gases extracted from cortex, stele, and roots of the 23–30 cm. region of each rhizome. Fig. 5 shows the plotted means. From the analysis of variance it appears that the change from Air Top/Air Bottom

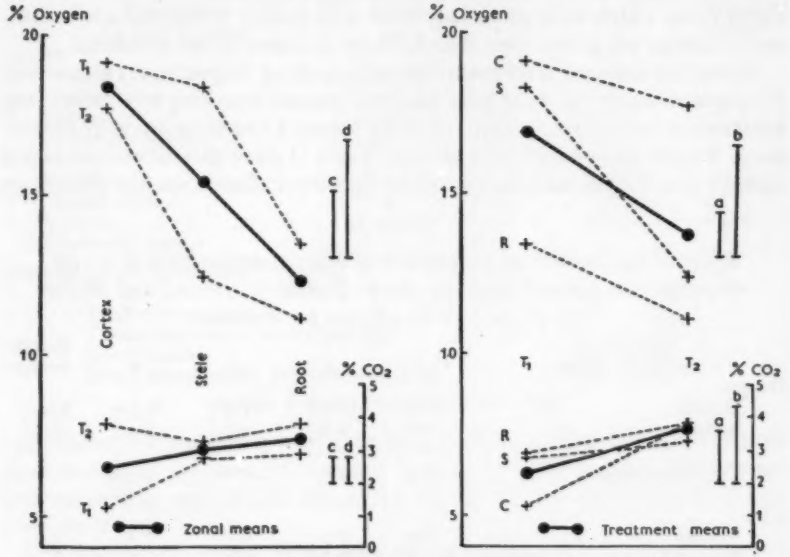

FIG. 5. Comparison of internal atmospheres of *Menyanthes* plants kept under conditions approximating to those of the gas chamber experiments of Section A. Means of three plants per treatment, sampled at 22·5 to 30 cm. level. T_1 = Air Top / Air Bottom; T_2 = Air Top / Nitrogen Bottom.

Least significant differences (5% level) are shown between: (*a*) zonal means, (*b*) zonal means under any one treatment, (*c*) treatment means, and (*d*) treatment means in any one zone.

conditions to Air Top/Nitrogen Bottom conditions had brought about a significant decrease in oxygen ($P = 0{\cdot}05$ to $0{\cdot}01$) and a less well marked increase in CO_2 concentration, significant only at the 0·1 level of probability. The stele showed a markedly significant decrease in oxygen, but for cortex only the differences in CO_2 are acceptable; it may, however, be remarked that the analyses of these data using a split-plot technique subjects 'treatment means' to a fairly stringent test, and that, on an ordinary '*t*' test, cortical differences in oxygen proved to be significant also. Details of the statistical methods employed will be found in Snedecor (1956) and Cochran and Cox (1955).

Effect of varying shoot length and number of leaves per shoot. It was thought that this would provide information on the effect upon internal gas levels of (*a*) increasing the length of the overall diffusion path and (*b*) varying the total surface area of the aerial shoot. If the magnitude of the resistance to the diffu-

sion of gases across the aerial surface of the plant is great relative to the total resistances encountered elsewhere in the plant, then the number of leaves present may be expected to influence the composition of the internal atmosphere.

The rhizomes of the experimental plants were sealed as before into long Pyrex tubes which were previously filled with freshly boiled and cooled culture solution; the plants were then held for 48 hours in the darkroom.

In the first series of determinations (27 July to 15 August 1951) plants with rhizomes about 75 cm. long were used and classed according to whether they possessed shoots with 1, 4, and 5 or more leaves; a fourth group were decapitated. Results from 6 replicates given in Table II show that differences in leaf number (see Treatment Means) had no significant effect upon the percentage

TABLE II

Effect of leaf number on composition of internal atmospheres of 75 cm. rhizomes. Gas sampled from two zones: Bottom (c. *70 cm.*) *and Middle* (*35–40 cm.*). *Six replicates per treatment*

Treatment, i.e. leaf number	5+	4	1	0	Zonal means
Oxygen					
Middle	8·8	11·1 ↑	11·0 ↑	10·5 ↑	10·4 ↑
Bottom	7·8	8·1 |	9·1 |	8·2 |	8·3 |
Treatment means	8·3	9·6	10·0	9·3	—
CO_2					
Middle	6·6	5·3 |	6·8 |	7·3 |	6·5 |
Bottom	7·2	7·1 ↓	8·6 ↓	9·9 ↓	8·2 ↓
Treatment means	6·9	6·2	7·7	8·6	—

Least significant differences between:

	O_2	CO_2
Treatment means	3·1	2·5
Treatment means in any one zone	5·4	4·0
Zonal means	1·1	0·7
Zonal means under any one treatment	1·7	1·0

Arrows indicate significant differences.

composition of gases sampled at the Middle (35–40 cm.) and Bottom (*c.* 70 cm.) regions. The overall mean difference between the oxygen levels of Bottom and Middle sampling regions was highly significant (see zonal means).

This experiment was repeated a week later with four replications in a run of only 5 days' duration. This time 50 cm. plants were used and Middle (22·5–30 cm.) and Bottom regions were sampled and gases were extracted from roots of the Middle region. Here again (see Table III) the number of leaves present could not be shown to affect significantly the composition of the internal gases at these two levels, nor of gases sampled from the proximal 15 cm. of roots taken from the Middle region. The differences shown between the three regions sampled were of the same order whatever the treatment involved, and although in all cases the mean values for Bottom oxygen were significantly lower than for Middle oxygen, it was only possible to distinguish

TABLE III

Effect of leaf number on composition of internal atmospheres of shorter (52·5 cm.) rhizomes. Gas sampled from two zones: Bottom (47·5 to 52·5) and Middle (22·5 to 30 cm.); root samples also taken from Middle zone. Four replicates per treatment

Treatment i.e. leaf number	4	1	0	Zonal means
Oxygen				
Middle	13·6	12·8	15·6	14·0
Middle roots	12·8	12·4	14·8	13·3
Bottom	11·7	10·5	12·6	11·6
Treatment means	12·7	11·9	14·3	—
CO_2				
Middle	4·6	4·7	4·1	4·5
Middle roots	4·4	5·6	4·6	4·9
Bottom	5·8	7·4	6·0	6·4
Treatment means	5·0	5·9	4·9	—

Least significant differences between:

	O_2	CO_2
Treatment means	2·7	1·5
Treatment means in any one zone	3·4	2·3
Zonal means	0·7	0·9
Zonal means under any one treatment	1·4	1·8

Arrows indicate significant differences.

a significant gradient between Middle and Middle Roots in terms of the overall zonal means. In the case of CO_2, zonal means showed a significant difference between Bottom and Middle Stem, but Middle Roots was indistinguishable from the latter.

The decapitation of the shoot gave rise to an oxygen concentration which was higher than in all other leafy plants. This increase, although consistent, was not large enough to be regarded as significant, and in any case could be explained in terms of the removal of the terminal zone of more metabolically active tissue. In view of these two experiments and their results it may be deemed unlikely that oxygen supply was held up to any extent during the experiments through variation in the leaf area exposed; bearing in mind that the experiments were conducted in darkness, it hardly looks as though stomatal closure is sufficient to impede oxygen movement to any appreciable degree.

From these figures, based upon plants all sampled within the space of a month, one may also derive evidence for the simple gradient of oxygen concentration which appears to exist down the rhizome. For example, if one brings together the means for one-leaved and for four-leaved plants shown in Tables II and III, making appropriate allowance for the innate variability of this material, a gradient can readily be discerned:

No. of plants	Zone (cm.)	Mean oxygen concentration: One leaf	Four leaves
4	23–28	12·8±1·1	13·6±0·7
6	35–40	11·0±1·7	11·1±0·9
4	48–56	10·5±1·4	11·7±0·9
6	69–75	9·1±1·2	8·1±0·8

This shows a mean concentration difference of between 3·5 and 5 per cent. of oxygen as between the upper-to-middle and lowermost zones of the rhizome, zones which are spaced some 45 cm. apart. It was not possible to demonstrate this gradient on the data available from the previously reported work. The figures also serve to draw attention to the presence of oxygen at the distal end of these 75-cm. long rhizomes at a mean concentration of 8 per cent., and it should be noted in this context that the concentration of the accompanying CO_2 in this zone is also at a level of about 8 per cent.

The effect of carbon dioxide concentration on the transmission of oxygen through the rhizome of bogbean. The apparatus of Fig. 1 was used, but with the topmost 25 cm. only of a decapitated plant which was enclosed for the greater part of its length within a glass tube of slightly greater diameter; the space between glass and plant was filled with mercury sealed in, top and bottom, by means of plasticine. Tube and plant were sealed into position between the upper and lower chambers of the gas-exchange apparatus. Thus movement of gas could only take place through the plant, and there could be no exchange of gas across the epidermis.

A 'head' of oxygen was established by filling the top chamber with air, and the bottom one with 10 per cent. of oxygen 90 per cent. nitrogen mixture. Movement of oxygen down this gradient could only lead to an increase of oxygen in the lower chamber if consumption by respiration during its passage through the plant was sufficiently low. After an initial period under these conditions, the gases were replaced with mixtures which maintained the same 'head' of oxygen, but in which the concentration of carbon dioxide in both chambers was raised either to 3 or 6 per cent.

The experimental procedure was as follows. The stem in its glass tube was fitted into the gas-exchange apparatus, and a freshly cut surface of stem was exposed at each end. After volume determinations of both chambers, air was flushed through the upper chamber for 15 minutes and 10 per cent. oxygen in nitrogen was simultaneously passed through the lower chamber. The taps were then closed, and apparatus and plant, kept in a bath in darkness at 22° C., was left for 24 hours, so that it might come into equilibrium with its surroundings. At the end of this time, top and bottom chambers were flushed through again with their respective mixtures, and after a short time for temperature equilibration, the manometers were adjusted, and the gases of both chambers were sampled and analysed, using a Haldane apparatus. After a further period of 24 hours, the gas in each chamber was well mixed, then sampled and analysed again. New gas mixtures, containing carbon dioxide at the chosen level but with oxygen in each case at the original level, were then introduced. Once again a day was allowed for equilibration, and the chambers were then recharged with the gas mixtures containing CO_2; these were then sampled and analysed. At the end of the subsequent 24-hour period further analyses were carried out on the gases of each chamber.

From these analyses, and knowing the original and final volumes of the upper and lower chambers, it was possible to calculate the volumes of oxygen

and carbon dioxide gained or lost by each chamber, and hence, by difference, the gas exchange of the 22·5 cm. length of rhizome used.

The results may be seen on the left-hand side of Table IV. Consumption of oxygen is low compared with Table II. These experiments were carried out between December and March; had they been carried out much later it is unlikely that it would have been possible to detect the movement of oxygen

TABLE IV

Data from experiment showing the effect of increasing CO_2 levels on the passage of oxygen through a 22·5 cm. length of rhizome of Menyanthes

CO_2 per cent.	Gaseous exchange in rhizome (in μl./hour/gm. fresh wt.) each set the mean of 4 plants		Overall increase of oxygen diffusing into lower chamber
	Oxygen intake	CO_2 output	ml. of oxygen
0	12·4±0·2	16·1±2·4	0·9±0·4
3	12·2±2·1	13·2±1·2	
0	12·4±0·7	12·0±0·8	2·5±0·7
6	6·3±0·7	4·8±0·9	

into the lower chamber. Whereas the change from 0 to 3 per cent. of CO_2 had no appreciable effect on gas exchange in the rhizome, the effect of changing from 0 to 6 per cent. CO_2 was quite marked.

The increase in the amount of oxygen passing through the plant to the lower chamber when the CO_2 level was raised, may be seen on the right-hand side of Table IV. The increase which results from raising the CO_2 concentration from 0 to 6 per cent. is nearly three times as great as when it was raised from 0 to 3 per cent., a difference which also has statistical significance ($P < 0{\cdot}001$). It is established therefore that CO_2, at concentrations of 6 per cent. or more, in such a way narcotizes the tissues of bogbean rhizome, that their oxygen consumption is reduced; under certain conditions this may allow more oxygen to diffuse through to the lowermost tissues beyond.

DISCUSSION

It is of interest to note that throughout this work there has appeared evidence of a seasonal variation in the internal gases of the rhizome. For example, in Air Top/Air Bottom plants, samples of gas taken in September from the zones at about the 30 cm. level were higher in oxygen by about 5–6 per cent. than in similar samples taken in May from Air Top/Aerated Culture Solution Bottom plants (data of Figs. 4 and 5). This feature was most marked in the rhizome, but was less apparent in the roots, where the difference was of the order of 2–3 per cent. It is, of course, arguable that more oxygen could have moved into plants which were fully exposed below to atmospheric oxygen, under the equivalent of chamber conditions, than into plants whose rhizomes

and roots were submerged in an aerated culture solution. At the same time, the first series of gas chamber experiments (see Fig. 2) showed that little more than one-fifth of the oxygen requirements of the plant was ever obtained in this way. Furthermore, if a comparison is made between *Air Top/Boiled Culture Solution Bottom* plants at the beginning and towards the end of the season, the same seasonal change is apparent.

The mean percentage oxygen concentrations for cortex, stele, and root at the 30–37·5 cm. level in such plants are shown below:

	Cortex	Stele	Root
5–19 May . . .	9·4±0·8	8·9±1·0	6·7±0·7
21 July–8 Aug. . .	14·4±0·9	14·0±0·7	11·3±0·8

(Six replicates for each period. Differences in all three zones are significant.)

It may well be that the rate of oxygen consumption by the developing shoot in the earlier part of the growing season results in a cutting down of the effective gradient of oxygen. This may mean that less oxygen is received by the lower, more distal, regions of the plant until such time as extension growth and leaf development have slowed down. This hypothesis seems also to be supported by the seasonal differences in the rates of oxygen consumption by tissue slices.

The investigations that have been described have shown that, in darkness, adequate supplies of oxygen may be expected to reach the lowermost tissues of *Menyanthes*, both in its rhizome and in its associated roots. In the light, as has been reported for other species (see for, example, Laing, 1940) a greater 'head' of oxygen may be built up in the leafy shoot which is reflected in increased concentrations below. Thus, in a parallel series of comparative determinations made on plants kept either in darkness with their rhizomes in well aerated water, or submerged in shallow tanks in full daylight, the following percentages of oxygen (mean values) were obtained:

	Cortex	Stele	Root
Sampling zone: 30–37·5 cm.			
Plants in light . .	17·6±0·6	16·2±0·9	14·7±1·7
Plants in darkness .	12·8±0·7	12·0±0·9	11·4±1·0

The highest cortical value obtained in the lighted plants at this level was 19·3 per cent., whilst darkened plants showed a maximum late-season value of 17·6 per cent. of oxygen in the cortex. With the rhizome under nearly oxygen-free conditions, cortical oxygen at this level rarely fell below 7 per cent. (mean value *c.* 12 per cent.); and root oxygen rarely fell below 4 per cent. (mean value *c.* 9·5 per cent.). Both are lowest at the beginning of the season. Even at a depth of 75 cm., mean values for mid-season determinations centre around 8 per cent., and in general oxygen concentrations are rarely so low that one might expect to encounter evidence of alcohol zymasis. It seems evident that this oxygen moves downwards in the airspace system along a straightforward physical diffusion gradient.

Furthermore, the data of the last section show clearly that any substantial

increase in the concentration of carbon dioxide in the airspace system results in a lowering in the rate of consumption of oxygen. This must in its turn lead to a flattening of the downward diffusion gradient in oxygen, since now more of this gas will be available to penetrate into the lower regions.

Movement of oxygen is thus limited in two main ways. It is limited physically by reason of the resistance to its diffusion which is occasioned by the very small pores which separate the large lacunae of root and rhizome. These are rarely more than 2–3 μ in maximum diameter; they are in other words of such dimensions that mass movement of gas cannot take place through the tissues as the result of applying a head of pressure. This will constitute a relatively constant rate-limiting factor, only to be modified as the result of local injury and flooding; and the length of the path, considered alone as a physical entity, can only serve to modify the time taken for the achieving of equilibrium conditions. It seems unlikely that resistance to diffusion at the surface of the plant contributes significantly to this factor.

Secondly, the movement of oxygen is limited metabolically in terms of its consumption by the tissues through which it is passing. The length of the overall diffusion path may obviously here be expected to influence the steepness of the diffusion gradient, since, for example, the total consumption of oxygen along the path will be approximately proportional to the amount of living tissue involved.

It has been made clear that this type of limitation will vary with the season, and will also depend upon the distance of the tissues concerned from the leafy shoot. It is to be expected that the Q_{O_2} will increase when the tissues are better ventilated, but it is equally worth noting that a long washing period also brings about a quite considerable increase (nearly fourfold) in the rate of oxygen consumption. This would strongly suggest that, either an inhibiting substance is being washed out of the tissues, or that, during this period, an established respiratory sequence (for the internal gas conditions prevailing prior to slicing) is being replaced by one more appropriate to fuller oxygen availability coupled with low CO_2 concentration. It may even be that these two possibilities are in some way linked together, and this is an aspect of the metabolism of *Menyanthes* tissue which is receiving more study.

Whatever turns out to be the position as regards its metabolism, *Menyanthes* has been shown to fall into the ecological category of those helophytes which, at least whilst they are in leaf, can supply adequate amounts of oxygen to their organs below water. The development of a large lacunar space within cortex and stele of this plant ensures a large reservoir of oxygen-containing gas. The level of this oxygen never seems naturally to fall to the point at which alcohol formation might constitute an embarrassing feature for the plant. This appears to be achieved by a process of auto-narcosis, whereby with increasing levels of CO_2 the consumption of oxygen falls off. Photosynthesis helps to boost the level of oxygen within the leafy plant. At present it can only be surmised that, during the winter months when leafless plants may become fully submerged, the green superficial cortical tissues of the rhizome may

contribute sufficient oxygen during light periods to maintain the levels which have been observed.

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The Effect of 2:4-Dinitrophenol on the Respiration of Apple Tissue in Relation to the Respiration Climacteric

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SUMMARY

The respiration of pre-climacteric apple pulp but not peel tissue can be stimulated by addition of DNP. Post-climacteric pulp and peel tissue cannot be stimulated by addition of DNP.

The disappearance of DNP stimulation of pulp tissue respiration, and also the development of a malate-decarboxylating system in the peel, are closely associated with the respiration climacteric in the whole fruit.

INTRODUCTION

It is possible that the climacteric in fruit respiration may be due to one, or both, of two causes. The pre-climacteric respiration level may be limited by some factor other than the respiratory enzyme level, which is either partially or completely removed at the climacteric, or new respiratory enzymes may be formed at the time of the climacteric.

Most of the recent theories of the causes of the climacteric in fruits come under the first category and suggest that the factor limiting respiration in the pre-climacteric stage is the rate of phosphate-ester formation which accompanies the respiratory oxidations. Hulme (1936) found that the climacteric in the English varieties of apples he used was always accompanied by an increase in protein, and he advanced the theory (Hulme, 1954) that the respiration rate of pre-climacteric apples is limited by the amount of phosphate-accepting substance (ADP) and that the climacteric protein synthesis releases new supplies of ADP from ATP, causing increased respiration. Pearson and Robertson (1954) independently suggested that respiration of pre-climacteric apples is limited by the amount of ADP available, but attribute the increased supply of ADP at the time of the climacteric to an increased demand of synthetic processes to maintain the protein content of the cells at that time, when, they suggest, increased protein breakdown occurs in their Australian apples. The latter workers also found that 2:4-dinitrophenol (DNP) stimulated the respiration rate of pre-climacteric apple tissue slices, and that this stimulation disappeared at the time of the climacteric. This finding, whilst in agreement with their explanation of the climacteric, could also be explained on the grounds of the formation of a natural uncoupling substance at the

climacteric; evidence in support of such an explanation for the Avocado pear had been reported by Millerd, Bonner, and Biale (1953).

Recently some evidence has been provided in support of the second possible cause of the respiration climacteric, namely the synthesis of new respiratory enzymes. Hulme (1954), in addition to the theory described above, also suggests that part of the protein synthesized at this time may consist of new respiratory enzymes, which further increase respiration. Recently Neal and Hulme (1958) have found that a new metabolic pathway, involving malate decarboxylation, develops in apple-peel tissue at the climacteric. Tager and Biale (1957) have also found that aldolase and carboxylase activity develops in banana tissue at the time of the climacteric.

The present work was undertaken with apple tissue slices to examine further both suggested types of 'climacteric mechanism'.

METHODS AND MATERIALS

Bramley's Seedling apples were used. The respiration-rates of single apples were measured at 12° C. by the Pettenkoffer method. For manometric experiments, peel tissue disks 1 cm. diameter were prepared at 1° C. as described by Neal and Hulme (1958). Pulp tissue slices, 1 cm. diameter, 2 mm. thick, were cut at 1° C. with a modified stainless steel cork-borer. Both peel and pulp disks were washed in 0·1 M. phosphate buffer (pH = 4·0) in a beaker, carefully dried on filter paper, and then 1 g. amounts of peel disks, or 2 g. amounts of pulp disks, were placed in the Warburg vessels. The direct method of Warburg was used. 3 ml. 0·1 M. phosphate buffer (pH = 4·0) were used as the suspending medium. There was no evidence of plasmolysis of the peel or pulp tissue using this concentration of buffer, and steady rates of oxygen uptake were obtained. The bath temperature was 28° C. Additions were made from the sidearms of the vessels after a steady rate of respiration had been attained. Added malate was neutralized to pH 4·0 with KOH.

RESULTS

The effect of DNP on respiration. The effect of a range of concentrations of DNP on the respiration rate of disks of pulp from pre-climacteric fruit was determined. It was found that respiration could be stimulated by concentrations between 8×10^{-7} M. and 8×10^{-6} M., with maximum stimulation at $3{\cdot}2\times10^{-6}$ M. This is close to the concentration found by Pearson and Robertson (1954) to give maximum stimulation with pulp tissue of pre-climacteric Granny Smith apples. It was also found that if glucose (0·1 M.) was added to the medium, the DNP stimulation was increased to about 1·5 times the control level. The control level of respiration was the same with phosphate-glucose and phosphate buffer alone, so phosphate-glucose solution was used for pulp-tissue studies. Concentrations of DNP above $2{\cdot}4\times10^{-5}$ M. were inhibitory to apple-pulp respiration.

No stimulation of respiration of pre-climacteric peel disks could be obtained by addition of DNP, either in the presence or absence of glucose, and

concentrations of DNP above 4×10^{-5} M. were inhibitory. No stimulation of respiration of post-climacteric peel and pulp tissue disks could be obtained by addition of DNP.

Fig. 1 shows typical results obtained by addition of $3{\cdot}2 \times 10^{-6}$ M. DNP to pre- and post-climacteric pulp tissue slices in the phosphate-glucose medium.

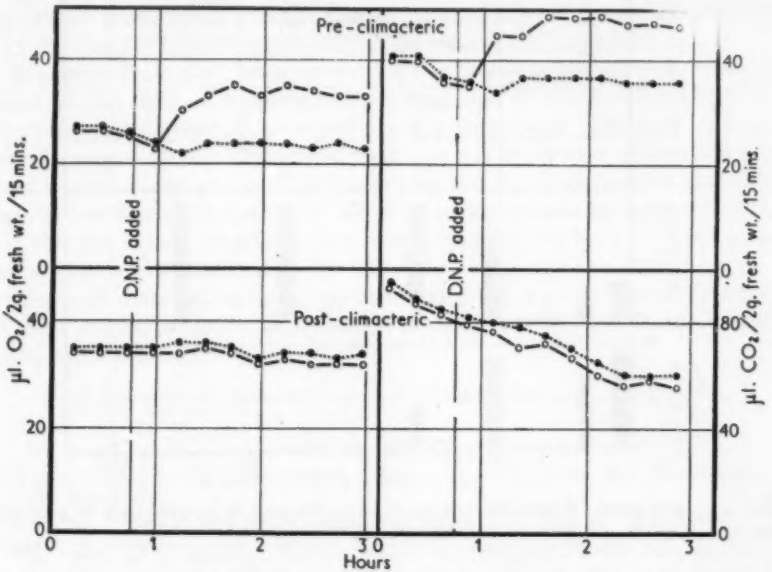

FIG. 1. Effect of addition of DNP on the O_2-uptake (left) and CO_2-output (right) of disks of apple-pulp tissue.

Control (—●—); (—○—) DNP.

The results also show that the pulp slices reflect the climacteric rise in respiration of the whole apple. The high initial rate of carbon dioxide production of post-climacteric pulp disks, falling steadily throughout the course of the experiment, is typical of the tissue at this stage.

The climacteric. An experiment was carried out to determine how closely, in time, the disappearance of DNP-stimulation of pulp-tissue respiration was associated with the respiration climacteric. At the same time, using the same fruit, the increase in carbon dioxide production resulting from addition of 0·1 M. malate to peel tissue was determined (see Neal and Hulme, 1958).

Apples were placed in store at 12° C. directly on picking, and the respiration rates of individual fruits were measured, the results being given in the upper part of Fig. 2.

At intervals, indicated by open circles on the respiration curves, two apples were removed from the respiration chambers, and the peel and pulp-tissue disks prepared from them were randomized for Warburg experiments. The

extra carbon dioxide evolved by the peel disks in 1 hour induced by addition of malate (0·5–1·5 hours after addition) is shown in the central part of the figure. The lower part of Fig. 2 shows the extra oxygen-uptake of pulp tissue after

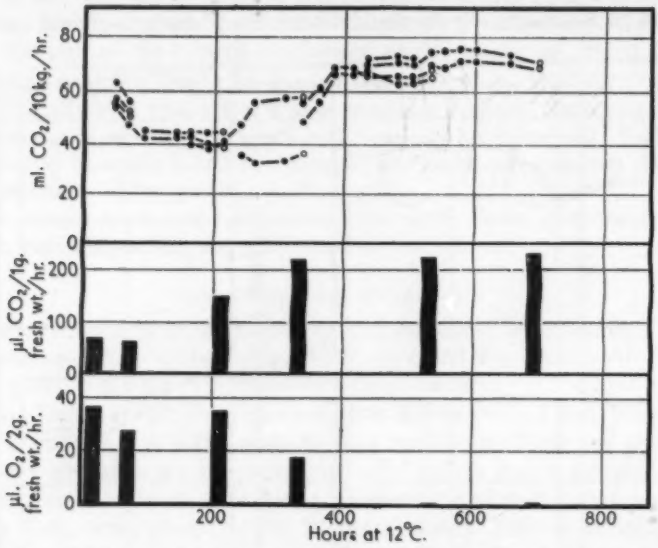

FIG. 2. Upper graph: Respiration rate of whole apples used to prepare disks of peel and pulp tissue for the experiments depicted in the middle and lower graphs.
Middle graph: Extra CO_2-output of peel disks to which 0·1 M. malate had been added.
Bottom graph: Extra O_2-uptake of pulp disks as a result of the addition of $3{\cdot}2 \times 10^{-4}$ M. of DNP

addition of DNP over the same period. Both malate and DNP stimulations were maintained, with only slight declines, over an experimental period of 2·5 hours after additions had been made.

DISCUSSION

It appears that both the development of the malate-decarboxylating system in the peel tissue and the disappearance of DNP stimulation of pulp tissue respiration are closely associated with the climacteric rise in respiration of the whole fruit. The observed development of the malate effect slightly in advance of the onset of the climacteric has been shown to be a result of the effect of the large difference in the temperature of the fruit from the store (12° C.) and the Warburg bath (28° C.). Near the onset of the climacteric the tissue is in a 'sensitive' state. Exact coincidence between onset of the climacteric and development of the malate effect occurs when the temperature of the Warburg is only 15° C. It appears likely, therefore, that the climacteric is caused both by disappearance of a respiration-limiting factor present in the pre-climacteric fruit (which DNP addition can remove) and also the development of new

respiratory enzymes. This is in agreement with the hypothesis advanced by Hulme (1954).

The R.Q.s of control and DNP-treated pre-climacteric pulp tissue are similar, approx. 1·4 (Fig. 1), suggesting that DNP accelerates the respiration without changing its metabolic pathway. The control level of oxygen uptake of post-climacteric pulp tissue is approximately the same as that of DNP-stimulated pre-climacteric pulp, suggesting similarity of the action of DNP and the changes occurring naturally at the time of the climacteric.

It appears from the present study that the increase in respiration brought about at the time of climacteric by a lessening of the 'braking-effect' of phosphorylating systems occurs only in the pulp tissue. The high carbon-dioxide output of post-climacteric pulp tissue is of interest concerning the development of the malate-decarboxylating system. It is thought that this high carbon-dioxide output may be due to malate released from the cut cells, and that the malate-decarboxylating system develops in both peel and pulp at the time of the climacteric.

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The Sporulation of *Penicillium notatum* Westling in Submerged Liquid Culture

I. THE EFFECT OF CALCIUM AND NUTRIENTS ON SPORULATION INTENSITY

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SUMMARY

The growth and sporulation of *Penicillium notatum* Westling in shaken submerged culture has been studied. Sporulation occurred only when calcium ions were added to the basal sucrose-mineral salts medium. Concentrations of 5 p.p.m. or less of calcium were ineffective and at least 35 p.p.m. was necessary for the maximum degree of sporulation. The replacement of calcium in the medium by strontium or barium enabled sporulation to occur, although these metal ions were less effective than calcium in that order. The calcium requirement for sporulation diminished as the total nutrient concentration was lowered and was similarly reduced if the concentration of the nitrogen source alone (sodium nitrate) was lowered, whereas reductions in the levels of other single nutrients caused relatively small alterations in the calcium requirement. Lowering the concentration of the nitrogen source caused increased sporulation in the presence of both limiting and non-limiting levels of calcium.

INTRODUCTION

At present there is little understanding of the process of sporulation in fungi. The influence of environment and nutrition on sporulation performance has often been studied, but little attention, however, has been paid to the analysis of metabolism or growth during sporulation. A recent book by Hawker (1957) surveys the physiology of fungal reproduction and defines many of its outstanding problems.

Foster *et al.* (1945) showed that conidia of *Penicillium notatum* formed in submerged (shaken) liquid culture were morphologically similar to those of surface culture. However, sporulation in submerged culture occurred only when high concentrations of calcium ion (0·5–5·0 per cent.) were present in the basal sucrose-mineral salts medium. Such shake cultures were chosen for the present investigation because they provide an artificial system with several features facilitating experimental analysis, viz. (i) only one spore type, (ii) simple sporing structures, (iii) homogeneous conditions throughout the culture, and (iv) easy separation of mycelium from medium.

MATERIALS AND METHODS

Stock cultures. These were derived from the *Penicillium notatum* strain, No. 419, in the culture collection of the Botany Department of Birmingham

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University; this strain came from the Baarn collection. To minimize variation, stocks were maintained in sterile soil cultures (Miller *et al.* 1947). Field soil in 2 oz. McCartney bottles was autoclaved 5 times at 15 lb. pressure for 45 minutes and after inoculation such cultures were incubated at 25° C. for 4 weeks and then stored at room temperature.

Inoculum. About 1 g. of soil from a stock culture was shaken with 10 ml. of sterile distilled water; 1 ml. of the supernatant was added to each of several bottles and dispersed over the inner walls, which had previously been coated with 2 per cent. malt agar by rolling the bottles as they cooled after sterilization. Spores formed in such cultures after 4–5 days at 25° C. were harvested in 0·1 per cent. v/v 'Teepol' solution and centrifuged at 2,000 r.p.m. for 2 minutes; the supernatant was discarded and the spores resuspended in sterile distilled water. Further washing was found to be unnecessary. The suspension was suitably diluted for inoculating media with the standard spore load of 5×10^5 spores per ml., which gave cultures attaining a maximum dry weight in approximately 72 hours.

Media. The basal medium (medium *F*) consisted of:—sucrose, 20·0 g.; $NaNO_3$, 6·0 g.; KH_2PO_4, 1·5 g.; $MgSO_4,7H_2O$, 0·5 g.; trace element supplement, 1·0 ml. (giving concentrations per litre of Fe, 0·40 mg.; Zn, 0·36 mg.; Cu, 0·08 mg.; Mn, 0·04 mg.; Mo, 0·04 mg.); distilled water to 1 litre. All chemicals used were B.D.H. 'Analar' grade.

Calcium chloride was added to medium *F* only when required for sporulation. Usually 1·0 g. of $CaCl_2$ per litre (360 p.p.m. of calcium ion) was added and such a medium is designated *F*Ca360. Media *F*Ca36, *F*Ca100, &c. have calcium ion concentrations of 36 p.p.m., 100 p.p.m., &c., respectively.

Incubation. Cultures comprising 25 ml. aliquots of medium in 100 ml. conical flasks were incubated at 25° C. and shaken at 125 cycles per minute over a stroke of 4 cm. which caused strong agitation without breaking the liquid surface. The degree of experimental variation was minimized by autoclaving and inoculating media in bulk before dispensing into individual culture flasks (Hadley, 1956).

Measurement of growth and sporulation. Unless otherwise stated samples consisting of 2 flasks each were taken every 8 or 12 hours for determination of dry weight and spore number. Cultures used for spore counts were blended for 2–4 minutes at 12,000 r.p.m. to free the spores from the pellet mycelia. Drops of this suspension were mounted on the two grids of a haemocytometer and 8 counts were made from each grid. Spore numbers were expressed as millions of spores per ml. of medium or, for comparing sporulation intensities in different treatments, as a 'sporulation index' which is the number of spores (in millions) per mg. dry weight.

EXPERIMENTAL RESULTS

A comparison of sporulating and non-sporulating cultures

Penicillium notatum sporulated in medium *F*Ca360 but not in medium *F*, confirming the observations of Foster *et al.* (1945) that calcium ions were

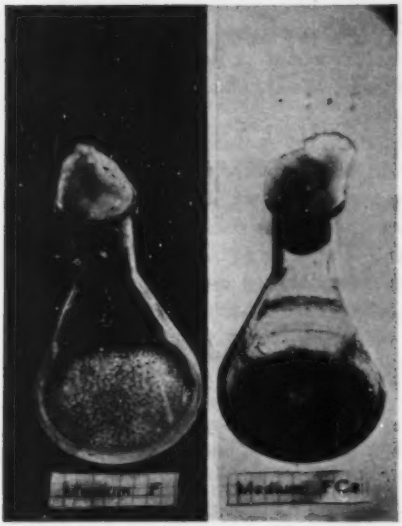

FIG. 1. Cultures of *Penicillium notatum* after 48 hours' growth at 25° C. in media *F* and *F*Ca360. The colonies in medium *F* are larger than those in medium *F*Ca360 and the dark colour of the latter is due to their intense sporulation.

FIG. 2. Colonies of *P. notatum* after 30 hours' growth at 25° C. in medium *F*.

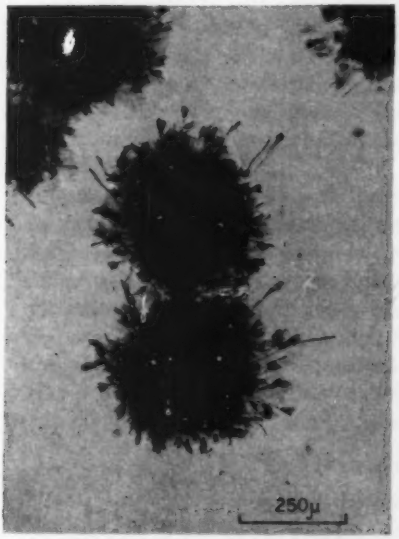

FIG. 3. Colonies of *P. notatum* after 30 hours' growth at 25° C. in medium *F*Ca360.

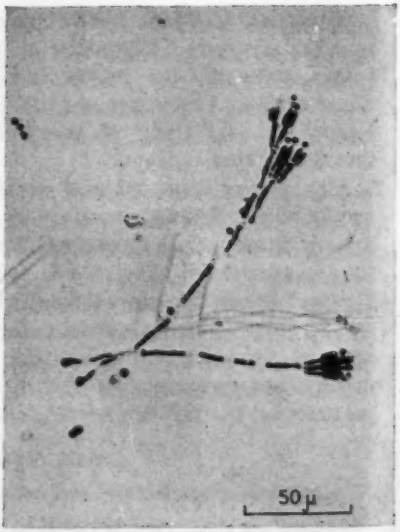

FIG. 4. Conidia formed in a shake culture of *P. notatum* germinating *in situ* to produce further phialides and conidia.

necessary for sporulation of this mould in submerged culture. The pellet mycelia (Fig. 1) typical of these cultures each arose from a number of spores and the presence of low concentrations of detergent did not prevent such clumping. After 48 hours at 25° C. in medium *F*, colonies had grown to 3 mm. in diameter with a mycelial edge of spreading little-branched hyphae (Fig. 2) whereas in similar *F*Ca360 cultures the colonies grew up to 1·5 mm. diameter

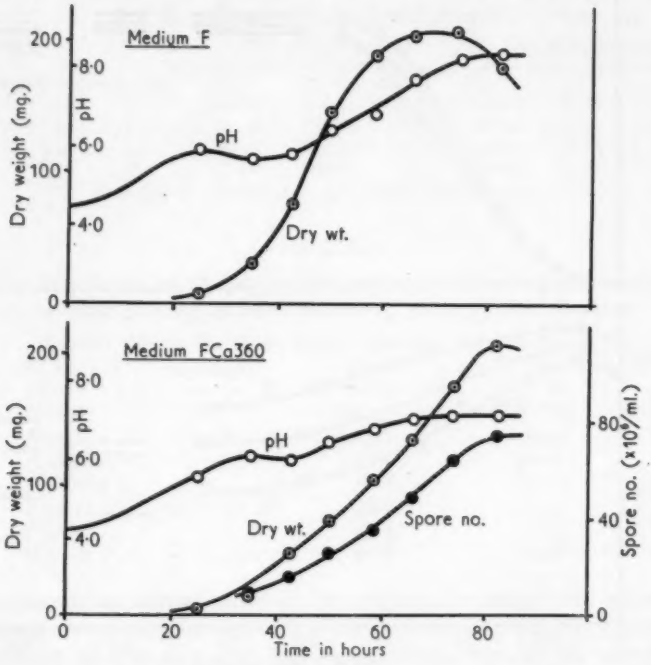

FIG. 5. The pH values (—o—), dry weights (—⊙—), and spore numbers (—●—) of shake cultures of *P. notatum* in media *F* and *F*Ca360.

with a dense mycelial edge of phialides and conidia (Fig. 3). These conidia germinated *in situ*, each developing a germ tube which immediately produced phialides (Fig. 4). Although colonies in medium *F* formed a few phialides, the number of spores in such cultures was negligible.

Dry weight, pH, and spore number determinations of cultures in media *F* and *F*Ca360 are given in Fig. 5 which shows that there is no marked difference between pH drift and dry weight in the two types of culture. Microscopic observation showed that spore production began 17·5 hours after inoculation in *F*Ca360 cultures, continued until growth ceased, and was more or less proportional to dry weight. A detailed study of the period of initial sporulation is reported in a later paper (Hadley and Harrold, 1958).

The influence of calcium, strontium, and barium ions on sporulation

The effect of calcium concentration on sporulation index and dry weight was measured after 60, 67, and 74 hours growth and is shown in Fig. 6. The sporulation indexes remained almost constant during the experiment although the dry weights were increasing. Cultures with 5 p.p.m. or less of added

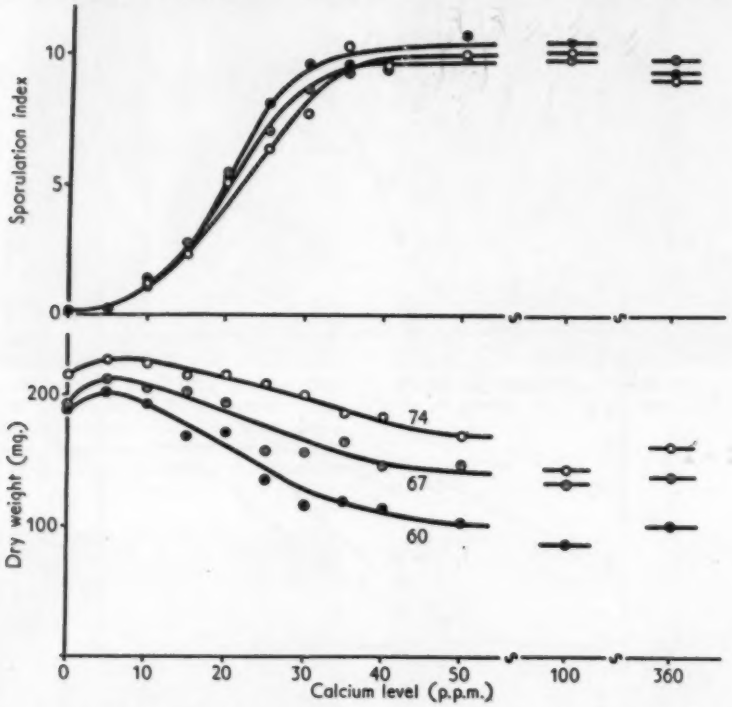

FIG. 6. The effect of calcium on the dry weight and sporulation index of *P. notatum* after 60 (—●—), 67 (—⊙—) and 74 (—○—) hours' growth.

calcium were virtually sterile, resembling those in medium *F*. Sporulation index increased to a maximum of about 10 as the calcium ion level increased from 5 to 35 p.p.m. and dry weight in this range was more or less inversely proportional to sporulation index. While increases in calcium concentration above 35 p.p.m. caused little change in sporulation index, the dry weights in such cultures decreased slightly. In medium *F*, therefore, *Penicillium notatum* requires the addition of 35 p.p.m. calcium ion for maximum sporulation.

The relative efficiency of calcium, strontium, and barium ions in promoting sporulation was measured after 54, 65, and 76 hours at levels of 20, 50, 100, and 400 p.p.m. The results from the 65-hour sample are typical and are given in Table I.

With each metal, the sporulation index of the cultures rose with increase in the ion concentration, sporulation in the calcium series being at a maximum with an ion concentration of 50 p.p.m. or more. Over the range tested, the order of decreasing sporulation activity was calcium, strontium, and barium.

TABLE I

The effect of calcium, strontium, and barium ions on the dry weight and sporulation of P. notatum after 65 hours' growth

Ion concentration p.p.m.	Dry weight mg.			Sporulation index		
	Ca^{++}	Sr^{++}	Ba^{++}	Ca^{++}	Sr^{++}	Ba^{++}
20	192·9	211·1	187·7	4·12	0·03	0·00*
50	124·8	185·3	183·2	9·79	0·24	0·00*
100	98·5	191·5	193·8	10·72	0·64	0·03
400	124·8	152·0	22·3	9·40	4·93	0·73

* A few spores present but insufficient to count.

TABLE II

The effect of calcium on the dry weight and sporulation index of cultures of P. notatum growing on basal medium of three different strengths

Medium	Sampling time	Dry weight mg.	Sporulation index
*F*Ca10	74 hours	200·1	1·27
*F*Ca20	,,	216·8	8·85
*F*Ca30	,,	193·4	12·65
F/2Ca5	74 hours	105·8	6·40
F/2Ca10	,,	107·0	10·91
F/2Ca15	,,	99·8	12·72
F/4Ca2·5	61 hours	48·9	5·54
F/4Ca5	,,	50·7	9·19
F/4Ca7·5	,,	41·4	9·38

With increasing concentrations of all three metals, dry weights fell as sporulation indexes increased. The highest barium level tested (400 p.p.m.) permitted a dry weight of only 22·3 mg., but this apparent toxicity did not inhibit sporing since the sporulation index at this concentration was the highest in the barium series.

The effect of nutrient concentration on the calcium requirement for sporulation

Media were prepared containing 10, 20, and 30 p.p.m. calcium ion (*F*Ca10, *F*Ca20, and *F*Ca30) to support low, moderate, and high degrees of sporulation respectively. They were also diluted to half strength (*F*/2Ca5, *F*/2Ca10, and *F*/2Ca15) and quarter strength (*F*/4Ca2·5, *F*/4Ca5, and *F*/4Ca7·5). The sporulation index and dry-weight maxima attained on these media are given in Table II and were reached after 74 hours in series *F* and *F*/2 and 61 hours in series *F*/4.

The dry weights decreased as the medium was diluted at all calcium levels tested, but the sporulation indexes behaved differently. A comparison of series *F* with *F*/2 shows that a half-dilution of medium increased the sporula-

tion index except at the higher calcium level where it was already at a maximum. Series $F/4$ resembled $F/2$ although its maximum sporulation index was only about 9. In further experiments where calcium levels were limiting sporulation, a reduction in total nutrient concentration always caused an increase in sporulation index whether the initial medium concentration was above or below that of medium *F*. The calcium requirement for maximum

TABLE III

The effect of different levels of single components of medium F on the dry weight and sporulation index of cultures of P. notatum, in the presence of limiting concentrations of calcium ion. Samples taken after 72 hours' growth

Nutrient level as fraction of content in medium *F*		Calcium content of medium in p.p.m.					
		10		20		30	
Control		Dry weight mg.	Sporulation index	Dry weight mg.	Sporulation index	Dry weight mg.	Sporulation index
Medium *F*		218·0	0·25	189·7	4·49	184·1	9·40
Sucrose	⅔	140·6	0·53	132·0	5·78	133·1	10·90
	⅓	57·0	1·55	57·1	9·12	57·8	16·60
$NaNO_3$	⅔	203·2	1·96	173·2	10·46	176·0	10·04
	⅓	147·4	12·80	138·7	15·15	136·2	15·77
KH_2PO_4	⅔	209·4	0·34	199·8	8·94	174·6	12·20
	⅓	189·5	0·54	196·4	11·01	168·8	14·08
$MgSO_4,7H_2O$	⅔	198·2	0·51	195·4	4·52	185·1	8·46
	⅓	200·7	0·33	195·0	4·46	179·7	8·90
Trace elements	⅔	201·4	0·76	206·2	5·60	188·3	9·62
	⅓	164·4	1·00	191·6	6·58	152·2	12·27

sporulation, therefore, apparently decreases as the nutrient status of the entire medium is lowered.

The nutrients sucrose, $NaNO_3$, KH_2PO_4, $MgSO_4,7H_2O$, and trace elements (treated as one nutrient) were tested, in turn, at two-thirds and one-third of their concentration in the control medium *F* to determine whether the observed dilution effect was associated with any single nutrient. Each nutrient concentration was tested at calcium levels of 10, 20, and 30 p.p.m., and the dry weights and sporulation indexes after 72 hours' growth in each of the 33 resultant media are given in Table III.

Apart from magnesium sulphate, a reduction in the concentration of each nutrient caused some increase in sporulation index. The previous experiment showed that a maximum degree of sporulation was maintained when the concentrations of both calcium and nutrients were equally reduced. If in the present experiment, therefore, a particular nutrient was likewise affecting the calcium requirement, then a high sporulation index would be expected in the presence of, for example, two-thirds of the normal concentration of that nutrient and 20 p.p.m. calcium, or 10 p.p.m. calcium and one-third of its normal concentration. This expectation is confirmed only in those media where the level of sodium nitrate is decreased. Thus, with the nitrogen level

at one-third normal and only 10 p.p.m. calcium, a sporulation index of 12·80 is reached, showing that it is the sodium nitrate level which markedly influences the calcium requirement for sporulation. Further experiments, not reported here, showed that the nitrate ion of sodium nitrate was responsible for this effect and that similar effects could be obtained with asparagine and other nitrogen sources.

The above results did not show clearly whether the reduction in nitrogen was enhancing sporulation merely by lowering the calcium requirement or

TABLE IV

The effect of various concentrations of sodium nitrate, expressed as multiples of the concentration of $NaNO_3$ in medium F, on the growth and sporulation of P. notatum in media containing low and high concentrations of calcium

	Calcium concentration			
	10 p.p.m.		360 p.p.m.	
Concentration of $NaNO_3$	Dry weight mg.	Sporulation index	Dry weight mg.	Sporulation index
×2·5	205·5	0·13	162·5	8·6
×1·0	202·3	1·8	150·0	11·3
×0·5	207·6	7·6	157·3	11·5
×0·1	57·0	23·0	63·3	19·9

whether it was also increasing the sporulation capacity of the mycelium. If the latter effect were also operative then an enhancement should be apparent with nitrogen level reductions in non-limiting as well as in limiting calcium concentrations. Table IV shows the result of 80 hours' growth on four sodium nitrate levels tested in two series of media, one having 10 p.p.m. calcium (limiting), the other 360 p.p.m. (non-limiting).

The sporulation index ranged from 0·13 to 23·0 in the 10 p.p.m. calcium series and, whether the sodium nitrate concentration was limiting growth or not, increased as the nitrogen level was reduced. Similar reductions of nitrogen level in the 360 p.p.m. calcium series increased the sporulation index in the same way but not to the same degree. Reducing the nitrogen level appears, therefore, to enhance the sporulation capacity of the fungus in addition to diminishing the calcium requirement for sporulation.

DISCUSSION

The nutritional role of calcium in fungi remains obscure although a few species are known to require this element for growth (see Hadley, 1956, for references). While calcium is known to promote asexual sporulation in some fungi (e.g. *Penicillium griseofulvum* and others, see Morton *et al.*, 1958) and is essential for perithecial formation in *Chaetomium globosum* and *C. brasiliensis* (Basu, 1952), its metabolic function in these systems is not known.

Unpublished experiments by one of us (G. H.) show that the sporulation of *P. notatum* in normal subaerial cultures on agar or static liquid media does not require the presence of calcium in the sucrose-mineral salts medium.

Since the sporing structures in subaerial and submerged cultures of *P. notatum* are morphologically identical it is presumed, on general grounds, that the differentiation processes leading to sporulation in both types of culture are likewise the same. It is probable, therefore, that the requirement for calcium is not an intrinsic part of the pattern of differentiation in this fungus but is specifically related to the conditions of submerged culture.

Sporulation of *Penicillium griseofulvum* (Morton *et al.*, 1958) in submerged culture is induced by several treatments, including the presence of calcium in the medium, but these are unnecessary for sporulation in subaerial cultures of this fungus. In subaerial cultures of both *P. griseofulvum* and *P. notatum*, therefore, the processes ending in sporulation are wholly induced and maintained by the interactions of aerial and submerged hyphae of the developing mycelium and the medium (which itself changes as growth proceeds) but in submerged cultures on the same media such interactions do not provide an adequate stimulus. In shaken submerged culture, although both fungus and medium change with time, all parts of the mycelium will be subjected at any given time to a similar environment whereas in subaerial culture the environment will be less homogeneous, e.g. the aerial hyphae which become conidiophores are not in direct contact with the medium; the peripheral hyphae experience different medium conditions from those affecting the older central parts of a colony. In submerged cultures of *P. griseofulvum* and *P. notatum*, therefore, it appears that the function of the different agencies inducing sporulation is to impart an element of heterogeneity into the system which stimulates mycelium, in some yet undefined way, to manifest the capacity for sporulation which develops during growth. The development of this sporulation capacity in *P. notatum* is considered in a separate paper (Hadley and Harrold, 1958).

In *Aspergillus niger* (Stokes and Gunness, 1946) the amino-acid composition of mycelium prior to sporulation differs from that found afterwards, indicating a considerable reorganization of nitrogenous constituents, and in *P. griseofulvum* Morton *et al.* (1958) suggest that 'absence of nitrogen is an important factor in inducing, or at least permitting, sporulation to occur', although this simple conclusion is complicated by some of their experiments in which sporulation proceeds in the presence of external assimilable nitrogen. Sporulation in *P. notatum* is probably also closely connected with nitrogen metabolism since reductions in the nitrate level of the medium cause marked changes in sporulation capacity and the calcium requirement for sporulation. However, in *P. notatum* the induction of sporulation cannot primarily depend on external nitrogen level since sporulation begins in 17·5 hours, during which time the nitrate level of the medium will not have been appreciably lowered by the small amount of growth (*c.* 0·5 mg. dry weight) which has occurred.

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The Sporulation of *Penicillium notatum* Westling in Submerged Liquid Culture

II. THE INITIAL SPORULATION PHASE

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SUMMARY

The initial sporulation time of *Penicillium notatum* Westling grown in shaken submerged culture inoculated with spores or vegetative mycelia is inversely proportional to the logarithm of the inoculum load, with a minimum time of 17·5 hours in the former and 8 hours in the latter cultures. The development of cultures is divisible into two stages. Firstly, there is a period of vegetative growth, the duration of which depends on the inoculum load, after which the culture can be described as mature. Calcium is not required for the development of this maturity. Secondly, the cultures when mature develop phialides and spores if calcium is added to the medium. The development of phialides and the first spores takes 6 hours from the time of adding calcium and this period is not influenced by the inoculum load of the culture. The medium from a mature culture promoted more rapid sporulation of vegetative mycelia placed in it than did a fresh medium, indicating the presence of a sporulation factor(s) in mature medium. Similar activity was also demonstrated in media which had supported growth of any one of five other *Penicillium* species or *Aspergillus niger*. The nature of the sporulation factor is so far unknown.

INTRODUCTION

In shaken submerged culture, *Penicillium notatum* produces large numbers of conidia continuously during its growth if calcium is present in the medium (Hadley and Harrold, 1958). The present paper is concerned with the changes which the mycelium and medium undergo in such cultures during the phase of initial sporulation.

MATERIALS AND METHODS

Details of the media and culture techniques used have previously been described (Hadley and Harrold, 1958). Medium *F*Ca360, used to induce sporulation, was the basal sucrose-mineral salts medium *F* to which 360 p.p.m. of calcium ion had been added (as $CaCl_2$). When vegetative mycelia were used as inocula they were obtained by growing shake cultures on medium *F* for 36 hours, aseptically removing the mycelia (*c.* 1 mm. diameter) and washing them by centrifugation in sterile distilled water.

EXPERIMENTAL RESULTS

Cultures inoculated with spores. The times of initial sporulation (first appearance of phialides with attached conidia) were determined in cultures with

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inoculum loads ranging from 10 spores to 2×10^6 spores/ml. These are presented in Fig. 1 together with the dry weights at the time of sporulation.

The initial sporulation time varied inversely with the logarithm of the inoculum load, falling to a minimum of 17·5 hours with a load of $1{\cdot}5 \times 10^5$ spores/ml. At the initial sporulation time, the dry weights of cultures within this inoculum range were always less than 0·5 mg. Increases in inoculum load up to 2×10^6 spores/ml. caused no further reduction in initial sporulation

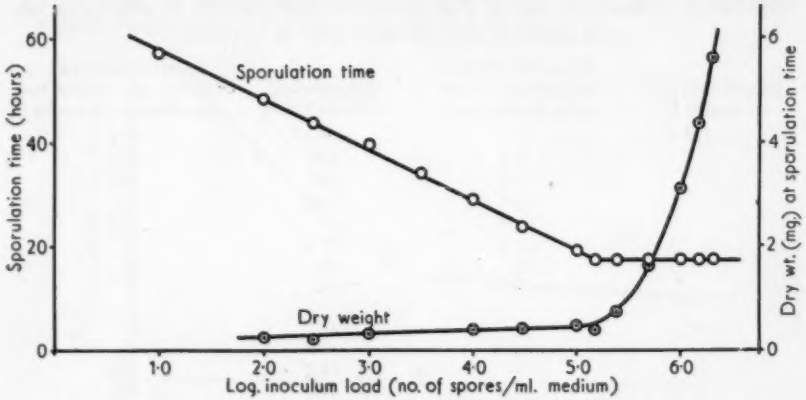

FIG. 1. The effect of inoculum load on the sporulation time (—o—) and the dry weight at this time (—⊙—) in shake cultures of *P. notatum* on medium *F*Cs360.

time so that the period of 17·5 hours appears to be a genetic minimum in cultures where the inoculum load is $1{\cdot}5 \times 10^5$ spores/ml. or more. The difference in dry weight of such cultures at the initial sporulation time is due to the fact that the amount of growth in a given time depends directly on the size of the inoculum.

Initial sporulation times were determined in media with calcium concentrations of 10 to 360 p.p.m. and in others where the levels of the carbon and nitrogen sources were varied, separately and together, from 0·05 to 2·5 times those in medium *F*. The sporulation time was not appreciably altered, however, in any of these media and it is therefore unlikely that the attainment of the particular mycelial condition necessary for sporulation is primarily determined by these factors.

The influence of calcium on sporulation was also examined by adding it to certain cultures at the time of inoculation and to others at intervals afterwards. The initial sporulation times of three series of such cultures, each with a different inoculum load, are recorded in Table I.

In each series, sporulation occurred at the predicted minimum time only if calcium had been added to the medium at least 6 hours before this time. When the calcium was added less than 6 hours before the predicted initial sporulation time then sporulation was delayed until 6 hours after the addition.

The requirement for calcium, therefore, appears to be confined to the 6-hour period immediately preceding initial sporulation and is associated with the differentiation of phialides and spores. Since this period is of the same duration in cultures with different inoculum loads, it must reflect only the time necessary for the differentiation of sporing structures and cannot be

TABLE I

The initial sporulation time of Penicillium notatum cultures to which calcium was added at various times after inoculation

Inoculum load spores/ml.	Time of calcium addition in hours after inoculation	Sporulation time in hours	Interval between addition of calcium and sporulation in hours
5×10^5	0	17·5*	17·5
	8·5	18*	9·5
	10·5	18*	7·5
	13·5	19·5	6
	15·5	21	5·5
	17·5	22·5	5
	33	39	6
	37	43	6
5×10^4	0	22*	22
	13	22*	9
	16	22·5	6·5
	18	24·5	6·5
	20	25·5	5·5
	22	27	5
	37·5	43·5	6
	41·5	47·5	6
5×10^3	0	40·5*	40·5
	13	41*	28
	16	41*	25
	18	41*	23
	37·5	43·5	6
	41·5	47·5	6
	45	51	6

* Minimum time of sporulation for this inoculum load.

related to the amount of growth during this time. Cultures to which calcium was added earlier than 6 hours before the predicted initial sporulation time never spored before this time, indicating that mycelium must attain a certain metabolic condition before sporulation is possible. A culture's development is, therefore, divisible into an initial calcium-independent phase of physiological differentiation, the length of which is a function of inoculum load, and a calcium-dependent phase of morphological differentiation of 6 hours' duration which is independent of the inoculum load. Mycelium which has passed through the phase of physiological differentiation may be described as mature but it cannot differentiate sporing structures unless calcium is present.

Aqueous extracts of mycelia and media from mature cultures were examined for sporulation activity. Such extracts, however, when autoclaved or Seitz-

filtered and added to cultures in media *F* or *F*Ca360 failed to initiate or accelerate sporulation. Since the sterilization procedures might have destroyed any activity present, further attempts were made to demonstrate activity by using twin-cell culture vessels in which sporing and non-sporing cultures were separated by collodion membranes or sintered-glass filters but without success. The search for sporulation factors was therefore pursued with the aid of cultures in which the inoculum consisted of vegetative pellet mycelia which could be conveniently added to or removed from flasks leaving the medium undisturbed for tests of sporulation activity.

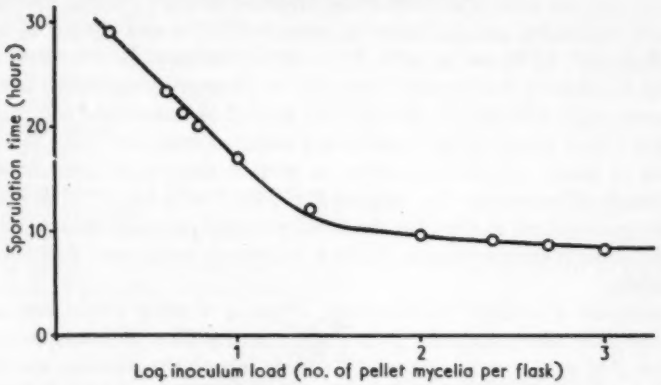

FIG. 2. The effect of inoculum load on the sporulation time of *P. notatum* inoculated with 36-hour-old pellet mycelia, in medium *F*Ca360.

Cultures inoculated with mycelium. The relationship between inoculum load and initial sporulation time of 36-hour vegetative mycelia inoculated into medium *F*Ca360 is recorded in Fig. 2. With inoculum loads between 5 and 25 mycelia per flask, the initial sporulation time is inversely proportional to the logarithm of the inoculum load but with larger inocula this time is constant at a minimum of 8 hours.

The relationship between inoculum load and initial sporulation time is similar, therefore, in both spore-inoculated and mycelium-inoculated cultures and the difference between the respective minimum times of 17·5 and 8 hours is equal to the time taken for spore germination in the first type of culture.

Cultures inoculated with five 36-hour-old mycelia per flask sporulated in about 22 hours and these were used to investigate the maturation process leading to initial sporulation. The mycelia in such cultures on medium *F*Ca360 were removed aseptically after 22 hours' growth (when sporulation was beginning) and the medium, undisturbed in the culture flasks, was reinoculated with five fresh 36-hour vegetative mycelia per flask; control flasks of fresh medium *F*Ca360 were similarly inoculated at the same time. The initial sporulation time in the latter was 24 hours, whereas the mycelia placed in the media which had supported sporulation produced their first

spores in only 8 hours. This result was repeatedly confirmed, demonstrating that in cultures developing towards initial sporulation the medium develops an activity which accelerates the sporulation of vegetative mycelia; media with such activity will be described as 'mature media'.

The influence of calcium on medium maturation was studied by comparing the initial sporulation times of mycelia in fresh medium *F*Ca360 with those in mature *F*Ca360, prepared as in the previous experiment, and in *F*(Ca360). Medium *F*(Ca360) is medium *F* which has supported the growth of five 36-hour vegetative mycelia for 22 hours at 25° C. and to which calcium (360 p.p.m.) has been added after the removal of the mycelia. Sporulation occurred within 8·5 and 9·5 hours in mature *F*Ca360 and *F*(Ca360) respectively but took 24 hours in fresh *F*Ca360. In medium *F*, therefore, in the absence of calcium, the growth of mycelia which remain vegetative nevertheless matures the medium in a similar way to that demonstrated with medium *F*Ca360. These results complement those obtained from the spore-inoculated cultures to which calcium was added at various times after inoculation and conclusively demonstrate that calcium is required only for the differentiation of sporing structures and not for the developmental processes which bring the cultures to the mature condition which is necessary before such differentiation is possible.

Maturation of medium by other fungi. Mycelia of other *Penicillium* species and *Aspergillus niger*, obtained from shake cultures after 36 hours' growth on medium *F* at 25° C. were examined for their capacity to produce sporulation activity in medium *F*. These mycelia were washed by centrifugation and, because their size varied with the species, the number inoculated into 25 ml. aliquots of medium *F* for maturation tests was varied in inverse proportion to their size so that each flask received approximately the same inoculum load (judged by eye) as the control flasks inoculated with 5 mycelia of *P. notatum*. After 24 hours' growth these cultures were filtered through sterile nylon, 25 ml. aliquots of each filtered medium were dispensed into sterile flasks containing 1 ml. of $CaCl_2$ solution (25 g./l.) and each flask was inoculated with five 36-hour mycelia of *P. notatum*. The initial sporulation times of *P. notatum* in these flasks and in flasks containing fresh *F*Ca360 are given in Table II.

The sporulation time of *P. notatum* was advanced in all media which had supported 24 hours' growth of the fungi tested. Except in the medium in which *Aspergillus niger* had grown, the test mycelia spored as soon as, or more quickly than, those in medium matured by *P. notatum* itself, but strict quantitative comparisons are not possible in this experiment because inoculum size was not rigorously standardized. It appears, therefore, that related fungi growing in medium *F* also produce factors which have sporulation activity for *P. notatum* similar to that developed by this fungus.

Properties of mature medium. Mature medium was obtained by growing five 36-hour mycelia of *P. notatum* in 25 ml. aliquots of medium *F*Ca360 for 22 hours at 25° C., filtering off the mycelia and collecting the medium in sterile flasks which had been cotton-plugged during autoclave sterilization.

The sporulation activity of media in such flasks, however, was greatly reduced unless they had been washed out with sterile water before receiving the mature medium. Medium collected in flasks autoclaved with 'Oxoid' metal caps instead of cotton plugs showed no loss of activity and such flasks were therefore used in subsequent tests. Experiments reported elsewhere (Hadley, 1956)

TABLE II

The effect of culture fluids of several fungi on the sporulation time of 36-hour old pellet mycelia of Penicillium notatum

Species used to mature medium	Sporulation time of *P. notatum* test mycelia in hours
*419 *Penicillium notatum* (control)	13
414 *P. italicum*	13
416 *P. spinulosum*	10·5
417 *P. luteum*	11·5
418 *P. thomii*	11·5
503 *Penicillium* sp. (of *frequentans* series?)	11
347 *Aspergillus niger*	14
Fresh medium *F*Ca360 (control)	20

* The number of the culture in the Mycology culture collection at the Botany Department, University of Birmingham.

TABLE III

The effect of heat treatment on the sporulation activity of mature medium

Medium	Sporulation time in hours
Fresh *F*Ca360 (control)	20
Mature *F*Ca360, untreated	13
" " boiled for 1 min.	13
" " steamed (100° C.) for 60 min.	*c.* 16
" " kept at 60° C. for 60 min.	*c.* 16
" " autoclaved for 15 min.	20

indicated that the inhibition of sporulation activity in the cotton-plugged flasks was probably due to small amounts of the stearate soaps incorporated in the cotton during its manufacture being released during autoclaving. The initial sporulation times of mycelia placed in mature media subjected to different heat treatments are given in Table III and show that the factors responsible for sporulation activity are thermolabile. Mature medium stored at 0° C. for 4 days showed a total loss of activity which suggests that the active factors are relatively unstable.

The identity of the sporulation factors in mature medium is unknown. Glucose-1-phosphate and fructose-1:6-diphosphate, reported by Hawker (1948) to stimulate perithecial formation in *Melanospora destruens*, gave negative results when tested for sporulation activity in the present system at concentrations ranging from 0·0001 to 0·01 per cent.

DISCUSSION

The initial sporulation time of cultures was inversely proportional to the logarithm of the inoculum load, falling to a minimum time of 8·5 and 17·5 hours in mycelium and sporé-inoculated cultures respectively. Since germination takes about 9 hours in spore-inoculated cultures, their post-germination sporulation time is comparable with the sporulation time in mycelium-inoculated cultures where the growing points are already organized at the time of inoculation. The significance of the logarithmic relationship is not clear but it is evident that initial sporulation time is related to the number of growing points present and that a definite amount of growth must occur before differentiation of sporing structures is possible.

Although the number of growing points in a culture will affect the rate of change in levels of nutrients and staling products, several facts indicate that these factors do not control the initiation of sporulation. Variations in the levels of carbon and nitrogen sources have little effect on initial sporulation time and it is unlikely that the small amount of growth (*c.* 0·5 mg. dry weight) preceding initial sporulation can appreciably alter the concentrations of nutrients or staling products. Furthermore, spores formed throughout the 3-day growth period of these cultures germinate and sporulate *in situ* (Hadley and Harrold, 1958) irrespective of the time they are formed and hence the particular concentrations of nutrients and staling products to which they are exposed.

Medium *F*Ca360, in which initial sporulation had occurred (mature medium) promoted a more rapid sporulation of vegetative mycelium than did fresh medium. Similar medium activity was also developed in equivalent cultures of medium *F* which remained vegetative, however, in the absence of calcium. During the brief period of growth preceding sporulation, therefore, substances (or a substance) are formed which influence the transformation of vegetative mycelia into a condition capable of sporulation and calcium is not required for the production of these substances. Experiments with spore-inoculated cultures showed that the period of initial sporulation is divisible into a preliminary phase of physiological differentiation, the duration of which depends on the inoculum load and a subsequent phase of morphological differentiation which lasted 6 hours, irrespective of the inoculum size. The first phase neither requires nor is influenced by calcium and is a period of maturation during which mycelium produces substances accelerating development towards the requisite physiological state for sporulation. The number of growing points present will affect the rate of accumulation of these postulated sporulation substances and it is suggested that the reduction of initial sporulation time with increase in inoculum load is related to this consequence of growth rather than alteration in the levels of nutrient and staling products. Once developed, the capacity for sporulation persists and becomes manifest only when calcium is added to the medium, whereupon sporing structures are differentiated within 6 hours irrespective of the time-length of the preceding maturation period.

The maturation period exhibited by *P. notatum* also exists in submerged cultures of *P. griseofulvum* where Morton *et al.* (1958) have shown that the 'distinct, although very small amount of growth' which is necessary before mycelium is capable of sporulation (their 'ripeness to spore') is achieved within 12–18 hours after inoculation; such mature cultures then responded to any one of several sporulation-inducing stimuli. It has been noted (Hadley and Harrold, 1958) that the stimuli inducing sporulation in submerged cultures of these two *Penicillium* species are not required for spore formation in normal subaerial cultures on the same medium. It is now apparent that these stimuli are not required, even in submerged culture, for the essential maturation process which precedes differentiation of sporing structures. The conditions of subaerial culture provide adequate stimuli for the completion of both physiological and morphological differentiation whereas in the more homogeneous environment of shaken submerged cultures the latter process is not achieved without the stimulus of certain additions to the medium or other special treatments.

The sporulation substances produced by *Penicillium notatum* are probably not specific metabolites of this mould alone because media in which any of five other *Penicillium* species or *Aspergillus niger* have grown also exhibited comparable sporulation activity. Until these sporulation substances and their sites of action have been characterized it is not possible to say whether they are related solely to the process of sporulation or have a more general metabolic significance.

ACKNOWLEDGEMENT

One of us (G. H.) wishes to record his appreciation to the Department of Scientific and Industrial Research for financial support during this work.

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Microbiological Synthesis of Fat

THE INFLUENCE OF PHOSPHATE CONCENTRATION ON THE METABOLISM OF *PENICILLIUM LILACINUM* THOM IN SURFACE CULTURE ON A SUCROSE MEDIUM

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SUMMARY

The influence of phosphate concentration in the medium on the relative amounts of fat, protein, and carbohydrate synthesized by *Penicillium lilacinum* Thom has been studied.

In a sucrose salts medium in which the C:N ratio was high (65:1) and conducive to fat synthesis, the carbohydrate content of the felt was decreased and the fat content increased when the concentration of sodium dihydrogen phosphate in the medium was raised from 0·73 to 1·46 per cent. w/v. In a similar medium in which the C:N ratio was low (65:2) this effect was not observed. In the medium of high C:N ratio with phosphate at the higher level, fat accumulated over a longer period than in any other case.

Felts grown with the high C:N ratio and with phosphate at the higher concentration were richer in protein and in total nitrogen than were felts developed with the high C:N ratio and less phosphate. This effect was still more pronounced in the medium of low C:N ratio. Change in the initial concentration of nitrate in the medium exercised more marked effects on the metabolism of the mould than did change in the initial phosphate level.

INTRODUCTION

THE intimate relationship between phosphate supply in the medium and fat formation in yeast was noted by early workers (Smedley-Maclean and Hoffert, 1923, 1924). Later work on this topic has been summarized by Kleinzeller (1948). In the present work *Penicillium lilacinum* has been employed, and the influence of phosphate at two levels, in conjunction with two ratios of carbon to nitrogen in the media, has been studied, with the object of ascertaining how the proportions of fat, protein, and carbohydrate in the mycelium were affected as growth proceeded.

EXPERIMENTAL

Strain. Penicillium lilacinum Thom obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, was used. It was maintained on slopes of the composition (g./l.): sucrose, 15; $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4,7H_2O$, 0·5; KCl, 0·5; $FeSO_4,6H_2O$, 0·01; agar, 15.

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Media and conditions of cultivation. The composition of the basal medium was (g./l.): sucrose, 170; K_2SO_4, 0·11; $MgSO_4,7H_2O$, 5·00; $ZnSO_4,7H_2O$, 0·05; $FeCl_3,6H_2O$, 0·16; made up with distilled water. Incorporation of $NaH_2PO_4,2H_2O$ at 7·3 g./l. and of $NaNO_3$ at 6·4 g./l. in this basal medium had been found by Philip and Walker (1958) to give a complete medium on which *P. lilacinum* could produce a yield of *c.* 50 per cent. of fat on felt weight. In the present work the basal medium was used with 6·4 and 12·8 g./l. of $NaNO_3$ and 7·3 and 14·6 g./l. of $NaH_2PO_4,2H_2O$, and the pH value of each was adjusted with NaOH to 6·8. These media were dispensed in portions of 50 ml. in 250 ml. Erlenmeyer flasks. After sterilization, each series was inoculated with a spore suspension and incubated at 25° during 16 days. At intervals of 6, 8, 10, 12, 14, and 16 days, there was withdrawn from each series a set of five flasks and the contents were analysed. The washing, drying, and grinding of mycelium and the subsequent estimations of residual sugar, and of the carbohydrate, fat, and nitrogenous contents of the felts, were conducted by procedures described by Naguib and Walker (1958).

RESULTS

Sugar utilization was highest in the medium containing both nitrate and phosphate at their respective higher levels but, from the 6th day onwards, the yield of mycelial felt calculated as a percentage on the weight of sugar consumed was higher in the two media of lower nitrogen content.

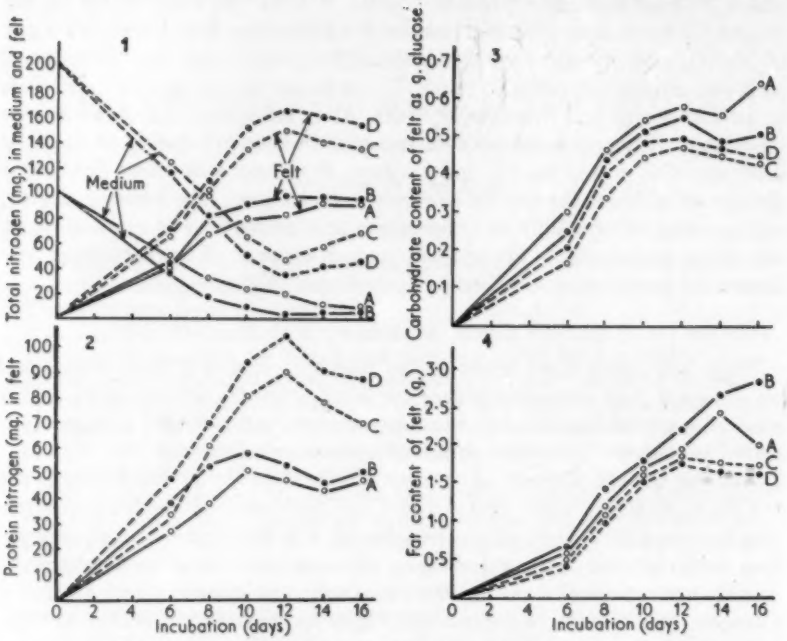
Nitrogen uptake. The rate of nitrogen absorption (Fig. 1) was greater in the two media of higher initial nitrogen level, and increase in phosphate concentration increased the rate at which the nitrogen was absorbed. Nitrogen absorption continued steadily in the medium of lower nitrate and lower phosphate content until the end of the experiment. In the two cases in which the initial nitrogen concentration of the medium was at the higher level, loss of nitrogen from the medium at 12 days gave place to a rise in its content of nitrogen, an indication that autolysis of fungal cells was in progress.

Protein content of the felts. Accumulation of protein (Fig. 2) proceeded steadily, reaching its maximum after 10 days in the cultures of higher C:N ratio and after 12 days in those of the lower C:N ratio. One result of increasing the phosphate concentration of the medium was clearly evident as an increase in the rate of protein synthesis, this effect being more pronounced in the medium of higher nitrogen content.

Carbohydrate content of the felts. Carbohydrate synthesis (Fig. 3) was highest when both nitrate and phosphate were at the lower level. With lower nitrate and higher phosphate the yield of carbohydrate was less, and it was less still when both nitrate and phosphate were supplied at higher level. The smallest quantity of carbohydrate was produced in the medium of higher nitrate and lower phosphate content.

Fat synthesis in the felts. The fat content of the felt (Fig. 4) was greater in the two media with the higher ratio of carbon to nitrogen (cf. Naguib and Walker, 1958). The highest of the four yields of fat was that synthesized in the

mycelium grown with the lower concentration of nitrogen in conjunction with the higher concentration of phosphate. In this last case fat synthesis was still proceeding when the experiment was terminated on the 16th day. In the media of lower C:N ratio, however, additional phosphate caused a slight depression in fat formation. The maximum percentage of fat in dry felt in the



FIGS. 1–4. Total N in medium and felts, and protein-N, carbohydrate, and fat contents of felts after times (shown as abscissae) in A, basal medium + $NaNO_3$, 6·4 g./l. + $NaH_2PO_4,2H_2O$, 7·3 g./l.; B, basal medium + $NaNO_3$, 6·4 g./l. + $NaH_2PO_4,2H_2O$, 14·6 g./l.; C, basal medium + $NaNO_3$, 12·8 g./l. + $NaH_2PO_4,2H_2O$, 7·3 g./l.; D, basal medium + $NaNO_3$, 12·8 g./l. + NaH_2PO_4 $2H_2O$ 14·6 g./l.

low nitrogen-low phosphate medium was 45·7, reached at 14 days. In the same period of time the low nitrogen-high phosphate medium afforded felt containing 49·3 per cent. of fat. The latter figure had increased to 52·7 per cent. by the 16th day.

DISCUSSION

In our experiments, the use of phosphate at its higher concentration raised not only the rate of sugar utilization but also that of nitrogen absorption and, consequently, the rate of growth. Our findings in this respect are in general agreement with those of Di Marco, Boretti, Julita, and Pennella (1955) who, in studies with *Streptomyces aureofaciens*, noted that the rate of consumption of sucrose was enhanced when additional phosphate was supplied to the

growing cultures. Earlier, Bichkovskaya (1940) had shown that in cultures of *Endomyces vernalis* phosphorus deficiency resulted in incomplete utilization of sugar.

The effects of keeping the initial nitrogen content of the medium at its lower level while at the same time raising the phosphate concentration were seen as a slightly greater accumulation of protein, a smaller accumulation of carbohydrate, and a raised yield of fat. The inverse relationship in the yield of carbohydrate to yield of fat noted here was not unexpected in view of the observations of Smedley-Maclean and Hoffert (1924) and of Kleinzeller (1948) on the role of phosphate in accelerating the conversion of carbohydrate to fat in various living cells.

A small reduction in the yield of fat was encountered when, in the higher nitrogen medium, lower phosphate was replaced by higher phosphate. In a similar connexion Bichkovskaya (1940) noted that excess phosphate in cultures of *Endomyces vernalis* caused diminution of fat yield, while Hesse (1949) has reported a case in which restriction of the phosphate supply in the medium used for a *Torula* species caused a reduction in the quantity of protein synthesized and a rise in the fat content of the cells.

The data reported in the present paper permit selection of experimental conditions in which the particular strain of *P. lilacinum* used in this work can produce in surface culture its highest yields of fat. It is also clear from the results of these experiments that a full picture of the extent to which phosphate concentration influences fat formation can only be obtained by studying the influence of phosphate in relation to the effects exercised simultaneously by the nitrogen level and by the particular C:N ratio in the medium.

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Nucleolytic Enzymes in Growing Root Cells

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SUMMARY

Experimental material consisted of fifteen serial sections 1·0 mm. in length, cut from the apex towards the base of bean roots of standard length. Optimum pH for ribo- and deoxyribonucleases were determined. Each enzyme was assayed at optimum pH in corresponding groups of sections, and the enzyme activity per cell calculated. Results are compared with those obtained using other tissues and with those of earlier investigations of the enzyme complement of the bean root.

INTRODUCTION

THE development of the enzyme complement in the cells of the bean root was studied in earlier investigations. Robinson and Brown (1952) showed that the activity of invertase, acid phosphatase, glycine oxidase, and dipeptidase increased during cell growth to a maximum level and then decreased. Decline in activity occurred when growth stopped, and it may be determined by the protein level of the cell. Activity of proteolytic enzymes also increases during cell growth, but the fall in activity occurs in mature cells after growth has ceased, and the level of other enzymes has begun to decline (Robinson, 1956). It has been suggested that the level of the proteolytic enzymes in the cell may be a factor which determines the level of certain other enzymes and hence of cell growth. Deoxyribonucleic and ribonucleic acids have been shown to play an important part in cell metabolism, and in protein synthesis in particular. It seemed of interest, therefore, to determine the activity of deoxyribonuclease and ribonuclease during cell growth.

MATERIALS AND METHODS

Experimental material consisted of 15 serial 1·0 mm. sections cut from roots of broad bean (var. Aquadulce). Techniques used for culturing beans and preparing sections were described in an earlier paper (Robinson and Brown, 1952).

Estimation of ribonuclease and deoxyribonuclease. Sections were incubated with either sodium ribonucleate or deoxyribonucleic acid. RNA or DNA present at the end of the experiment was precipitated. The supernatant consisted of hydrolysis products which were estimated as inorganic phosphate after mineralization.

Interpretation of experimental results is based on the assumption that quantity of substrate hydrolysed is proportional to quantity of enzyme. This assumption is only valid if two conditions are fulfilled: that rate of reaction

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remains constant with time, and that reaction rate is proportional to enzyme concentration.

The course of the enzyme reactions was studied in the preliminary experiments. Comparable groups of 20 sections from the region 4·0–5·0 mm. from the root apex were incubated in 2·0 ml. of either 1 per cent. RNA or 2 per cent. DNA for varying periods of time at 35° C. Mean values for three experiments with each substrate are given in Table I.

TABLE I

Time course of hydrolysis of 1 per cent. RNA or 2 per cent. DNA by twenty sections 4·0–5·0 mm. from root apices

Time in hrs.	0·5	1·0	1·5	2·0
RNA Total P. mg. $\times 10^{-5}$/sect./hr.	56	58	61	60
DNA Total P. mg. $\times 10^{-4}$/sect./hr.	32	34	36	34

It will be seen that the rate of hydrolysis remains constant throughout the 2-hour period, and other experiments showed that the rates per section were the same when section number per 2 ml. of substrate varied between 5 and 20.

The optimum pH for activity of ribo- and deoxyribonucleases in the bean root were not known. Comparable sets of 20 sections were each placed in 2·0 ml. of RNA dissolved in buffer solutions of varying pH values. Phthalate sodium hydroxide buffers were used for pH 3·0–6·0 and barbiturate (Michaelis, 1930) for pH 7·0–9·0. A similar series of experiments was performed using DNA dissolved in 0·1 N. sodium acetate and pH adjusted to required value with hydrochloric acid.

The details of the technique for estimating ribonuclease which incorporate the results of the preliminary experiments are given below. After freezing treatment (Robinson and Brown, 1952) 20 1·0 mm. sections were incubated in 2·0 ml. 1 per cent. sodium ribonucleate (Schwartz Labs. N.Y.) dissolved in phthalate buffer pH 6·0 for 4 hours at 35° C. The sections were then removed by filtration and the filtrate made up to 10·0 ml. with distilled water. Unused RNA in a 5·0 ml. aliquot of filtrate was precipitated with 5·0 ml. 0·25 per cent. uranyl acetate dissolved in 2·5 per cent. trichloroacetic acid (Kunitz, 1940). The precipitated RNA was removed by filtration after 30 minutes. Total phosphate in the supernatant, which is a measure of ribonuclease activity, was estimated by the method of Kuttner and Lichenstein (1930) after heating 0·5 ml. of supernatant with 0·35 ml. of concentrated sulphuric acid for one hour. The inorganic phosphate in the supernatant, which gives a measure of the extent of the breakdown of RNA, was also estimated.

In the case of deoxyribonuclease 20 sections after freezing treatment were placed in 2·0 ml. of 2 per cent. DNA (Schwartz Labs. Inc. N.Y.) in 0·1 N. sodium acetate adjusted to pH 7·0 with N. HCl, and incubated for 2 hours at 35° C. The sections were then removed by filtration and the filtrate made up to 10·0 ml. with distilled water. 2·0 ml. of filtrate were added to 2·0 ml. of 5 per cent. trichloroacetic acid and 0·4 ml. 6N. HCl (Schmidt and Thann-

hauser, 1945) and the precipitated DNA removed by filtration after 1 hour. Inorganic and total phosphate were estimated in the same manner as for ribonuclease. Values given in the next section are the means of five observations.

Inhibition of RNA'ase and DNA'ase. Inorganic phosphate may be produced directly by the enzymic breakdown of the nucleic acids, or from the end products of nucleolytic enzyme action. The substance $53D_k$ (Rogers and Spensley, 1954) inhibits nucleolytic enzymes, but has no effect on bean-root phosphatase. Total inorganic phosphate was estimated in the solution after sections were incubated with 1·0 ml. 0·2 per cent. $53D_k$, and 1·0 ml. of either 2 per cent. RNA or 4 per cent. DNA.

Expression of results. Quantities are given as either mg. phosphorus (inorganic or total) produced per section per hour, or per cell per hour. The average number of cells in each section was estimated by means of the cell-maceration technique of Brown and Rickless (1949).

RESULTS

Ribonuclease: effect of pH on activity. Results given in Table II show a sharply defined optimum at pH 6.

TABLE II

Effect of pH on liberation of phosphates from RNA

20 sections 3·0–4·0 mm. from root apices in 2·0 ml. RNA

pH of substrate	3	4	5	6	7	8	9
Inorg. P. mg. $\times 10^{-5}$/sect./hr.	0	3	10	14	13	11	9
Total P. mg. $\times 10^{-5}$/sect./hr.	7·5	16	46·5	78	36·5	28·5	30·5

Enzyme activity of the intact root. Mean values for inorganic and total phosphorus released by successive sections incubated with RNA at pH 6·0 are shown graphically in Fig. 1 B. Values for inorganic are much lower than those for total phosphorus. Ribonuclease activities per cell in the several sections are shown graphically in Fig. 1 A. Activities of the two apical segments are approximately equal, there is a twelvefold increase from the second to the twelfth section, and thereafter activity remains approximately constant.

Deoxyribonuclease: effect of pH on activity. Results are given in Table III and show that the pH optimum for release of inorganic phosphate is 6 and for organic 7·0; in both cases it is not as sharply defined as with RNA.

TABLE III

Effect of pH on liberation of phosphates from DNA

20 sections 3·0–4·0 mm. from root apices in 2·0 ml. DNA

pH of substrate	3	4	5	6	7	8	9
Inorg. P. mg. $\times 10^{-4}$/sect./hr.	0·04	2·1	4·0	4·25	4·0	3·5	1·75
Total P. mg. $\times 10^{-4}$/sect./hr.	5·1	15·0	28·0	30·0	34·0	25·0	12·0

Enzyme activity of the intact root. Mean values per section are plotted in

Fig. 2 B and per cell in the several sections in Fig. 2 A. The latter curve is similar to that for ribonuclease.

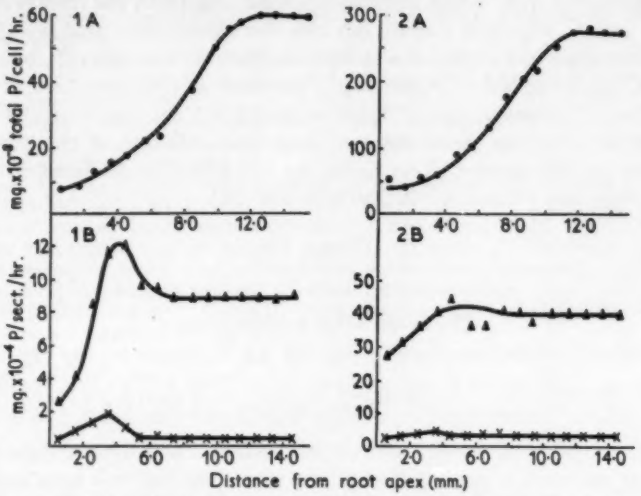

FIG. 1. A. Ribonuclease activity per average cell. B. Ribonuclease activity per section. ▲—▲ total phosphate, X—X inorganic phosphate.

FIG. 2. A. Deoxyribonuclease activity per average cell. B. Deoxyribonuclease activity per section. ▲—▲ total phosphate, X—X inorganic phosphate.

Effect of inhibitor. In the presence of $53D_K$ neither RNA nor DNA is broken down and no inorganic phosphate is produced.

DISCUSSION

The results of this investigation may be compared with those for nucleolytic enzymes in other tissues, and also with those for other enzymes in the bean root. Ribonuclease in bean-root sections has an optimum pH of 6·0 which is near to that (pH 6·2) found by Schlamowitz and Garner (1946) using extracts from soya bean; the deoxyribonuclease value of 7·0 is the same as that recorded for purified enzyme from beef pancreas (Laskowski, 1951).

$53D_K$ inhibits the release of both inorganic and organic phosphates when bean roots are incubated with either RNA or DNA. Bean-root phosphatase is not inhibited by $53D_K$. It would appear therefore that the inorganic phosphate is released from the end-products of RNA'ase and DNA'ase action, and not directly from RNA or DNA. Similar results were obtained by Schlamowitz and Garner (1946) using crude soya bean extracts incubated with RNA. One enzyme, ribonucleinase (= ribonuclease) produced acid-soluble phosphate, and a second, phosphomonoesterase inorganic phosphate. The substrate for the phosphomonoesterase was not investigated, but it is significant that activity was considerably higher when crude RNA containing mononucleotides was used.

In earlier investigations it was shown that the activity per cell of all enzymes studied, except dipeptidase, either remains constant or decreases at the extreme tip of the root. The activity of RNA'ase and DNA'ase remain constant in this region. Maximal activity per cell for dipeptidase, acid phosphatase, glycine oxidase, and invertase is at 8·0 mm. from the root apex (Robinson and Brown, 1952) and for two proteolytic enzymes at 10·0 mm. and 12·0 mm. respectively (Robinson, 1956). RNA'ase and DNA'ase with maximal activity per cell at 12·0 mm. from the root apex resemble one of the proteolytic enzymes in this respect. The similarity is further emphasized if relative activity per unit protein is calculated (Table IV).

TABLE IV

Relative enzyme activity per unit protein

(The lowest value is taken as unity)

Section	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
DNA'ase	1·3	1·0	1·7	1·6	2·2	2·2	2·9	2·7	3·4	3·3	4·1	4·4	4·4	4·6	4·4
RNA'ase	1·0	1·1	2·3	2·8	2·6	2·5	3·0	3·0	5·0	5·1	6·1	6·3	6·2	6·0	6·1

Relative enzyme activity per unit protein of both nucleolytic and proteolytic enzymes increases to approximately 12·0 mm. from the root apex and thereafter remains constant, whereas for the other enzymes studied increase is to 7·0 mm. only.

Activity of the nucleolytic enzymes may be considered in relation to the nucleic acid content of the cells. Nucleic acid estimations were made by Holmes, Mee, Hornsey, and Gray (1955) using experimental material identical with that of this investigation except that the growing zone of the root was slightly longer. They found that the concentration of ribonucleic acid phosphorus (RNAP) was higher than deoxyribonucleic acid phosphorus (DNAP) in all cells. Five regions of growth—meristematic, transition, early, late, and fully elongated—were defined. Concentration of both RNAP and DNAP per cell increased from the tip to the early elongation zone, then RNAP increased very slightly to the fully elongated zone, whereas DNAP decreased. Activity of RNA'ase per cell was higher than that of DNA'ase, and Holmes *et al.* found a higher concentration of RNAP than DNAP. Over the major portion of the growing zone concentration of both nucleic acids is increasing in the cell, as is the enzyme activity. The concentration of RNAP falls and DNAP levels out in cells which are not fully elongated. Maximum enzyme activity occurs in fully elongated cells, and therefore synthesis of substrate ceases slightly before that of enzyme. A similar feature was also shown by the proteolytic enzymes (Robinson, 1956). It may be that at certain stages of development concentration of substrate may be one of the factors influencing enzyme level.

It is a pleasure to record our thanks to Dr. H. Rogers of the National Institute of Medical Research, Mill Hill, for the gift of a specimen of $53D_K$, and to Mr. C. H. Ramsden for technical assistance.

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Phosphorylated Compounds in Plants

II. THE ESTIMATION OF HEXOSE PHOSPHATES AND ADENOSINE PYROPHOSPHATES IN PLANT TISSUE BY THE METHOD OF SLATER

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SUMMARY

Modifications of the method of Slater for the estimation of hexose phosphates and adenosine pyrophosphates in plant tissue are described. Triphosphates of nucleosides other than adenosine interfere with the estimation of adenosine pyrophosphates in plant tissue, but other interference is not serious if the concentration of hexose phosphates or adenosine pyrophosphates is not lower than about 0·15 μmoles per ml. of tissue extract.

INTRODUCTION

To study the concentration of hexose phosphates and adenosine pyrophosphates in plant tissue in physiological experiments (Rowan, Seaman, and Turner, 1956; Rowan and Turner, 1957) a method was required which would estimate these compounds in crude extracts of tissue in concentrations lower than 1 μmole per ml. The enzymic method of Slater (1953) was preferred to ion-exchange chromatography which did not separate adenosinediphosphate (ADP) from adenosinetriphosphate (ATP) when applied to unpurified extracts of pea seedlings (unpublished data). Although several types of interference occurred when unpurified extracts were analysed by Slater's method, they did not prevent accurate analyses in most extracts examined. This paper describes the types of interference found, and the methods used to overcome them.

MATERIALS AND METHODS

Reagents. ATP was supplied by Pabst Laboratories and Sigma Chemical Co. *Uridinetriphosphate* (*UTP*), *guanosinetriphosphate* (*GTP*) and *guanosinediphosphate* (*GDP*) were supplied by Pabst Laboratories. *Uridinediphosphate* (*UDP*) was prepared from UTP by the method described by Rowan (1957). *Glucose-1-phosphate* was prepared by Mr. E. T. Whitmore by the method of Hanes and Hill (unpublished work). *Glucose-6-phosphate* was supplied by Schwartz Laboratories and British Drug Houses Ltd. *Fructose-1:6-diphosphate* (*HDP*) was prepared by Mr. E. T. Whitmore using the method of Neuberg, Lustig, and Rothenberg (1943). *Reduced diphosphopyridinenucleotide*

(*DPNH*) supplied by Boehringer & Sons (Mannheim) was made up to 10^{-3} M. in 0·02 M. glycylglycine buffer, pH 7·6.

Enzymes. Enzymes A and B were prepared by the methods of Racker (1947) and Slater (1953). It was found necessary to check the pH at various stages of the preparation. Enzyme A was diluted twentyfold with ice-cold water before use. *Hexokinase* supplied by Pabst Laboratories was dissolved to give a 1 per cent. (w/v) solution in 1 per cent. (w/v) glucose, and stored at −15° C. *Myokinase* was prepared by the method of Colowick and Kalckar (1943).

The preparation of extracts for analysis. The tissue samples were extracted at 1° C. in 1·0–1·5N. $HClO_4$ in a pestle and mortar or a Waring 'Blendor'. The water content of a replicate sample of tissue was determined. Extracts were clarified by filtration or centrifugation, adjusted to pH 7·6 with 4N. KOH, and the precipitate containing $KClO_4$ removed after 1 hour at 1° C. (Kratzing and Narayanaswami, 1953). The neutral extract was stored at −15° C.

THE ENZYMIC PROCEDURES

These were essentially as described by Slater (1953).

The analysis of HDP: Procedure A.

$$HDP + 2DPNH + 2H^+ \xrightarrow{\text{(Enzyme A)}} 2 \text{ glycerolphosphate} + 2DPN^+ \quad (1)$$

The analysis of hexose monophosphate (HMP) (Procedure B (a)) or of reactive phosphate (∼P) (Procedure B (b)).

$$HMP + {\sim}P + 2DPNH + 2H^+ \xrightarrow{\text{(Enzymes A and B)}} 2 \text{ glycerolphosphate} + 2DPN^+ \quad (2)$$

HMP is the sum of the concentrations of glucose-6-phosphate, glucose-1-phosphate, and fructose-6-phosphate. ∼P is the sum of the concentrations of the β- and γ-phosphate-groups of ATP and ADP.

The analysis of ATP: Procedure C.

$$\text{Glucose} + ATP \xrightarrow{\text{(Hexokinase)}} \text{glucose-6-phosphate} + ADP \quad (3)$$

Reagents were added to the tissue extracts to give final concentrations as follows: glycylglycine buffer, pH 7·6, 0·02 M.; $MgCl_2$, 0·007 M.; glucose, 0·0075 M. This solution is called the 'reaction mixture'.

Procedure B (*b*) was used to follow the decrease in ∼P after the hexokinase reaction, as the high concentration of HMP in plant extracts prevented samples large enough for accurate analysis being taken. The procedure used by Slater was modified in the following way: 0·02–0·3 ml. of hexokinase was added to 1·0 ml. of reaction mixture held at 25° C., and the reaction stopped at the time required by adding 0·1 ml. 12N. $HClO_4$. The tubes were chilled in an ice bath and adjusted to pH 7·6 with a known volume of 3N. KOH. $KClO_4$ was removed after chilling for 1 hour at 1° C. Slater (1953) removed

the precipitate of hexokinase by centrifugation before neutralization to prevent interference with Procedure B (*a*). Interference with Procedure B (*b*) by small amounts of hexokinase is negligible (Slater, private communication). Duplicate analyses (Tables I and IV) were made on separate hexokinase digestions.

Table I shows that the concentrations of ATP measured with reaction times of 10 and 20 minutes were not significantly greater than those measured with 5 minutes.

TABLE I

The analysis of ATP by Procedure C using different reaction times

Concn. of ATP: (M. × 10^4)

Reaction time (min.) Expt.	5	10	20	Volume of hexokinase (ml.)
1	4·11	4·19	4·35	0·1
	4·15	—	—	—
2	4·21	—	4·42	0·1
	4·41	—	4·15	—
3	3·63	3·67	3·71	0·1
4	1·56	—	1·57	0·1
	1·62	—	1·65	—
5	1·77	—	1·79	0·02

Therefore, we can conclude that the hexokinase reaction is complete in 5 minutes in these conditions, and the ADP formed is not hydrolysed by an apyrase contaminating the hexokinase. For this reason, it is not necessary to add fluoride to the reaction mixture to inhibit such a hydrolysis.

INTERFERENCE

The analysis of authentic hexose phosphates or adenosine pyrophosphates by the method of Slater (1953) is unambiguous provided the enzymes required for Procedures A and B are sufficiently active. For this reason, occasional analyses of standard solutions of authentic compounds are desirable, particularly for Procedure B (*b*). When extracts of plant tissues are analysed, compounds in the extracts interfere with the analyses in several ways, which are described below.

Procedures A and B. The graph of extinction plotted against time shows two stages (Fig. 1). The first represents the rapid, enzymic oxidation of DPNH; the second is a slow oxidation of DPNH by an unknown agent, thought to be flavoprotein by Slater (1953) or denatured haemprotein by Morton (private communication). If a sample of an authentic compound and an extract of plant tissue are analysed at the same time, Stage 1 of the plot for the extract is often longer than that for the pure compound (Fig. 1). Although most of the perchlorate is removed from the extract as the insoluble K salt, the amount remaining appears to be sufficient to cause this inhibition. In

some experiments, when samples of 0·5 or 0·6 ml. were analysed by Procedure B (*b*), the concentration of ~P+HDP measured was lower than that when the volumes taken were smaller. Interference of this type is difficult to detect, as Stage 1 of the graph is prolonged to such an extent that it appears to pass into Stage 2. When it is necessary to analyse such large volumes, it is advisable to test the recovery of a known amount of ~P added to the sample.

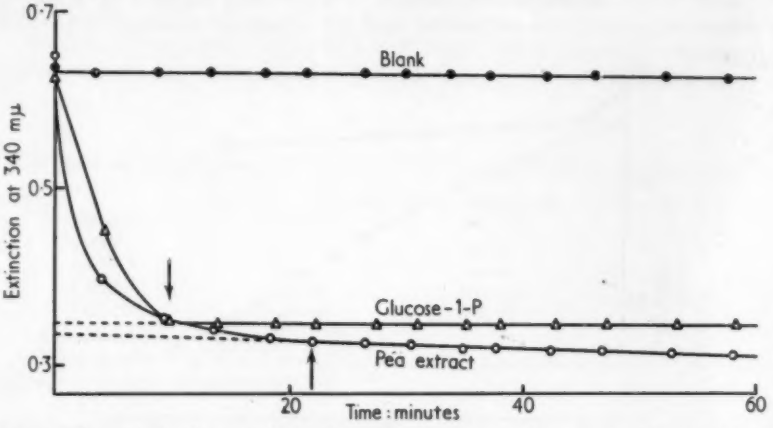

FIG. 1. The analysis of a sample of glucose-1-phosphate, and an extract of pea seed for HDP+HMP by Procedure B (*a*). The arrows show the points of transition from Stage 1 to Stage 2.

The enzyme fractions used in Procedures A and B contain enzymes other than those required for the reactions concerned in the analyses. These enzymes may slowly catalyse the oxidation of DPNH by compounds other than dihydroxyacetone phosphate (see Slater (1953): Eqn. 3) or slowly form the substrate for Reactions A or B from substances in the extract. Slater (1953) has described interference with Procedures A and B (*a*) and (*b*) by α-ketoglutarate, and with Procedure B (*a*) by hexose, fructose-1-phosphate, and polysaccharide. The result of such interference is that the slope of Stage 2 for the experimental cuvette is steeper than that for authentic compounds (Fig. 1). To show that the extrapolation is made from Stage 2, and not from Stage 1 extended by inhibition of the enzymes, the analysis is repeated using varying amounts of enzymes.

When extracts containing HMP and ~P in excess of HDP are analysed for HDP, sufficient of the enzymes catalysing Procedure B are present in Enzyme A to interfere with the analysis. Adding ethylenediaminetetraacetic acid (0·01 M.) to the cuvettes effectively inhibits the reactions of Procedure B (Slater, private communication). When HMP is estimated by Procedure B (*a*) in extracts of fruit using enzyme concentrations normally used in Procedure B (*b*), the rate of DPNH oxidation sometimes increases 5 or 10 minutes after the start of the reaction. This interfering reaction is lessened by using more

dilute enzymes. Fig. 2 shows the plots for analyses of an extract of pear fruit using both concentrated and dilute enzymes. Interference of this type has occurred in extracts of the fruit of apple, pear, tomato, and banana. As it does not occur in Procedure B (*b*), it is likely to be due to a reaction in which hexosemonophosphate is produced, rather than one in which DPNH is oxidized by an unknown reaction.

Slater (1953) describes the method of correcting analyses of HDP in presence of pyruvic and oxaloacetic acid. No significant amounts of pyruvic

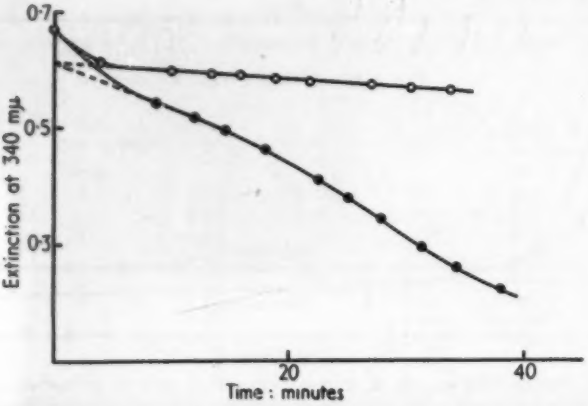

FIG. 2. Interference of Procedure B (*a*) by high concentration of enzymes in the analysis of HDP+HMP in an extract of pear fruit (0·1 ml.). Enzyme A diluted 5-fold, B 25-fold, curve —●—; enzyme A diluted 20-fold, B 100-fold, curve —○—. All enzyme volumes were 0·1 ml.

acid have been found in extracts of pea seed, maize roots, or apple fruit, and it is probable that the α-keto-acids are unstable when tissue is extracted by the method described above. The concentrations of oxaloacetic acid (up to 0·01 μmoles per g. fresh weight) detected in plant tissue by Isherwood and Niavis (1956) would not be detected in Procedure A.

Ling and Lardy (1954) have shown that the affinity of UTP for phosphohexokinase extracted from muscle is equal to that of ATP. Thus, the γ-P group of any UTP in the extracts would be included in the analysis of ~P by Procedure B (*b*) (Eqn. 2). The reaction of GTP with phosphohexokinase has not been described. Analyses of samples of authentic UTP, UDP, GTP, and GDP show that only the γ-P groups and not the β-P groups are included in analyses of ~P (Table II).

The system of enzymes catalysing the reaction

$$2\text{UDP} \leftrightharpoons \text{UTP} + \text{uridine-5'-phosphate} \qquad (4)$$

has been extracted from yeast and animal tissue (Strominger, Heppel, and Maxwell, 1954; Herbert, Potter, and Takagi, 1955; Lieberman, Kornberg,

and Sims, 1955), but neither these enzymes nor enzymes catalysing the reaction

$$2\text{GDP} \leftrightharpoons \text{GTP} + \text{guanosine-5'-phosphate} \qquad (5)$$

are present in the enzymes used for Procedure B.

UTP has been identified in young pea seedlings (Rowan, 1957) and tomato fruit (unpublished work), and both UTP and GTP in wheat plants (Bergkvist, 1956), fresh pea seed (Rowan and Turner, 1957), and banana fruit (unpublished work).

If the enzymes become slightly turbid when added to cuvettes containing tissue extracts, a cuvette containing tissue extract but no DPNH is used as

TABLE II

The analysis of samples of UDP, UTP, GDP, and GTP by procedure B (b)

Compound	Amount of sample* (μmoles)	~P by analysis (μmoles)	Groups of ~P per molecule
UDP	0·0456	0·0012	0
UTP	0·0450	0·0421	1
GDP	0·0578	0·0017	0
GTP†	0·0609	0·0491	1

* Calculated from extinction coefficients.

† This sample of GTP was shown to contain an appreciable amount of GDP when examined by paper chromatography.

reference for the analysis. The blank value is determined in the usual way. When reference cuvettes containing banana-fruit extracts were compared with blank reference cuvettes, increases in optical density up to 0·03 units were found.

Non-enzymic oxidation of DPNH by the plant extracts is detected by measuring the extinction at intervals before enzymes are added to the cuvettes. Severe interference of this type has been found when analysing large volumes of fruit extracts (0·5–0·6 ml.). It is overcome by reducing the volume of extract taken for analysis.

Procedure C. Berg and Joklik (1953) reported that preparations of hexokinase contained nucleosidediphosphokinase, and that transfer of phosphate from UTP to glucose in the presence of hexokinase could occur through the following reaction:

$$\text{UTP} + \text{ADP} \xrightleftharpoons{\text{(nucleosidediphosphokinase)}} \text{ATP} + \text{UDP} \qquad (6)$$

and the reaction shown in Eqn. 3. Catalytic amounts of adenosine pyrophosphates were assumed to be present in the UTP used by Kornberg (1951).

To examine the affinity of hexokinase for UTP and to see if the hexokinase contained nucleosidediphosphokinase, the transfer of the γ-P group of UTP to glucose was compared using a sample of UTP purified by paper chromatography (Pabst lot 704) and a sample containing a trace of ATP (Pabst lot 701). Table III shows that the transfer of γ-P groups of UTP was slower than that from ATP (see Table I), but was stimulated by a trace of ATP. Hence the hexokinase probably contained nucleosidediphosphokinase.

The sum of the concentration of ATP and UTP can be measured in Procedure C by using a concentration of hexokinase greater than that required to analyse ATP. Table IV shows the results from an experiment designed to determine the conditions required.

TABLE III

Percentage of γ-P transferred in 5 and 20 minutes in the hexokinase reaction to glucose from samples of UTP containing ATP and purified by paper chromatography

	5 minutes			20 minutes		
ml. Hexokinase . . .	0·02	0·1	0·3	0·02	0·1	0·3
UTP containing ATP (lot 701) . . .	35	73	94	75	94	97
UTP purified (lot 704) .	7	19	52	15	48	89

TABLE IV

The recovery of authentic UTP added to a reaction mixture containing extract of pea seed, using Procedure C with 0·3 ml. hexokinase at 25° C.

(Quantities expressed as M. × 10^{-4})

	~P+ HDP (1)		UTP* added (2)	Reaction time (min.)	~P after hexokinase reaction (3)		ATP+† UTP (4)	UTP‡ recovered (5)
Reaction mixture A	5·87 / 5·94	5·91	1·11	5	3·53 / 3·62	3·58	2·33	1·28
	—		—	10	3·57 / 3·55	3·56	2·35	1·16
Reaction mixture B	4·89 / 4·72	4·80	—	5	3·76 / 3·73	3·75	1·05	—
	—		—	10	3·68 / 3·53	3·61	1·19	—

* Calculated as A−B, column (1).
† Calculated as column (1)−column (3).
‡ Calculated as A−B, column (4).

The figures in column (5) show that as all UTP added was recovered, the analysis of UTP was complete in 5 minutes, using 0·3 ml. of the hexokinase solution. Similar experiments on recovery of GTP have not been carried out, but as the percentage transfer of the γ-P group of authentic GTP to glucose was similar to that of UTP, it is probable that the effect of GTP in tissue extracts will be similar to that of UTP.

It is possible to reduce the error in estimating ATP in an extract containing UTP by reducing the amount of hexokinase used to the minimum required to analyse ATP. This must be determined by measuring the recovery of authentic ATP added to a tissue extract, as extracts slightly inhibit the hexokinase reaction. Thus, only 57 per cent. of authentic UTP added to an extract

of pea seed was included in an analysis of ATP, using 0·02 ml. hexokinase and a reaction time of 10 minutes. As the concentration of UTP in plant extracts is likely to be small compared with that of ATP (Bergkvist, 1956; Rowan, 1957; Rowan and Turner, 1957), the concentration of ATP measured using a reduced amount of enzyme will be little higher than the correct value.

EXPERIMENTAL VARIATION

The stability of stored extracts. It is known that the phosphate bonds of ATP are hydrolysed on storing as a solution (Bailey, 1949). To test the extent

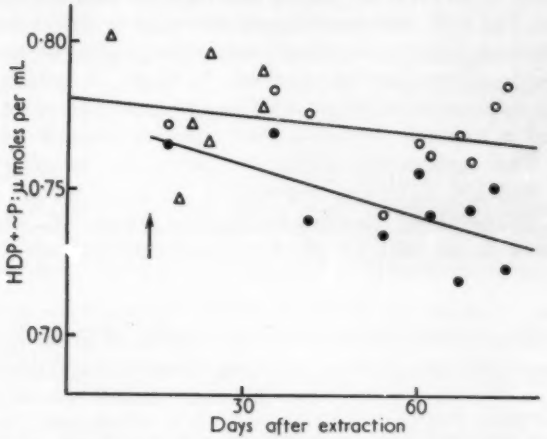

FIG. 3. The results of analyses of HDP+~P by Procedure B (*b*) on an extract of pea seed stored at both −15° C. and at room temperature. o extract stored at −15° C.; Δ reaction mixtures containing the extract stored at −15° C.; ● extract stored at room temperature. The arrow indicates the time of removal of the sample to room temperature.

of this decomposition, an extract of pea seed held at −15° C. was analysed for HDP+~P at intervals for over 2 months. Fourteen days after the extract was prepared, a sample was stored at room temperature over chloroform and analysed in the same enzymic run as the sample held at −15° C. The results of the analyses are shown in Fig. 3. The concentration of HDP analysed on day 74 was 0·034 μmoles per ml. The analyses were carried out on 0·1 ml. samples. The decomposition of HDP+~P calculated from the equations for linear regression of concentration on time was 0·025 per cent. per day at −15° C., and 0·072 per cent. per day at room temperature.

Standard error. The mean difference between duplicate analyses made in a single enzymic run (0·0007±0·0003 μmoles) was significantly lower than the mean for separate runs (0·0016±0·0008 μmoles) at the level $P = 0{\cdot}01$. The changes in extinction from which the standard errors were calculated were, with two exceptions, greater than 0·150 units. However, from inspection

there was no positive correlation between the mean change in extinction and the difference between duplicates, and the standard errors are considered independent of the mean change in extinction. The standard error calculated from the analyses over the period of 2 months on the extract of pea seed stored at −15° C. (Fig. 3) was 0·0016 μmoles.

The recovery of acid-labile phosphates added to a tissue extract

Replicate samples of pea seed were prepared by selecting 10 pea pods containing at least 5 mature seed. Two samples of 20 seed (A and B), weighing about 10 g., were prepared by placing two alternate seed from each pod in each sample. The fifth seed in each pod was used to determine the water content of the seed. Sample A was then extracted by grinding with acid-washed sand in a pestle and mortar with 10·0 ml. N. $HClO_4$, to which was added 1–3 ml. of a solution of authentic ATP or glucose-1-phosphate. Sample B was extracted in a similar way, with water added in place of the solution of phosphate. The supernatant solutions collected by centrifugation were neutralized with 4N. KOH and analysed in duplicate by the appropriate procedures. All operations described were carried out at 1° C. The recoveries measured were 82 per cent. for glucose-1-phosphate and 100 per cent. for ATP.

DISCUSSION

This paper has shown that the enzymic method of Slater (1953) can be used to measure the concentration of hexose phosphates and adenosine pyrophosphates in extracts of plant tissue. The most serious form of interference comes from the γ-P groups of 5′-triphosphates of nucleosides other than adenosine which are included entirely in the analysis of ~P. Only about half the concentration of nucleoside triphosphates other than ATP (which is likely to be small compared with ATP) is included in the analysis of ATP. The amounts included in the analysis would be reduced further if the hexokinase used in the determination of ATP contained no nucleosidediphosphokinase (Berg and Joklik, 1953).

If we assume that the concentration of ATP is greater than that of other triphosphates present, an approximation to the concentration of ADP can be calculated from the expression ~P−(Total γ-P)−ATP. The concentration of ADP thus calculated will be low by the amount of the other γ-P groups included in the analysis of ATP. Hence the ratios of ADP/ATP calculated from such analyses may be seriously low. However, the concentration of ~P and total γ-P can be measured accurately, and provide valuable data in physiological experiments. Apart from interference from nucleoside-5′-triphosphates other than ATP, severe interference has occurred only when the concentrations of compounds in the extract were low and the extinction of the extracts at 340 mμ was high.

The underestimation of ~P in Procedure B (*b*) is hard to detect and it is advisable to add a sample of a standard solution to a tissue extract and measure recovery. For two reasons, failure of this kind is thought to be due

to low activity of myokinase in the enzyme preparations: (1) adding a separate preparation of myokinase in addition to Enzymes A and B restored the activity of an inactive preparation; and (2) using enzymes from the same preparation, the activity is adequate for analyses of HMP by Procedure B (*a*) but not for analyses of ~P by Procedure B (*b*).

As the decomposition of the organic phosphates in neutralized tissue extracts held at −15° C. is very slow, serious errors do not occur if there is unavoidable delay in making the analyses required. The decomposition is little greater in extracts held at room temperature.

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Changes in the Distribution of Pectin Methylesterase across Leaf Abscission Zones of *Phaseolus vulgaris*

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SUMMARY

Determinations of pectin methylesterase in abscission zones and surrounding parts of Phaseolus leaves have shown a relatively high activity in young tissues, which decreases as the leaf grows older. The greatest fall occurs in the pulvinus.

In non-senescent leaves, the enzyme activity is higher in pulvini than elsewhere and there is a gradient of activity across the line of abscission from the pulvinus to the petiole or stem. During senescence this gradient falls, and is generally reversed at abscission. Data suggest that the advent of abscission may be linked to the steepness of this gradient.

If abscission of leaves or isolated abscission zones is accelerated by appropriate treatments there is a more rapid decrease in pectin methylesterase activity than in the controls, and a more rapid fall in the gradient across the line of abscission. If abscission is retarded the enzyme activity is sustained and the gradient is maintained or increased.

The results are discussed in relation to the changes that may occur in the pectic constituents of cell walls during senescence and abscission, and in relation to some of the known effects of auxins on pectin metabolism.

INTRODUCTION

THE chemical defoliation of cotton plants before mechanical harvesting of the bolls has become an accepted practice in many parts of the United States. The problems associated with this procedure have stimulated numerous investigations both physiological and anatomical into the processes involved in leaf abscission, and much is now known of the chemical and environmental factors which control leaf fall, and of the structural changes which may occur in the tissues.

Considerably less is known of the enzyme processes associated with abscission or of the changes in activity which may take place in these systems during the separation of the cells. It is generally accepted that pectic enzymes must be involved since dissolution of cell wall material is an essential part of the process.

The present paper describes a study of the distribution and changes in pectin methylesterase (PME) activity during natural and artificially accelerated or delayed abscission in *Phaseolus vulgaris*.

EXPERIMENTAL METHODS

Material. Plants of *Phaseolus*, variety Canadian Wonder, were grown in soil and sand under normal greenhouse conditions. Only the primary leaves, from the stage of their unfolding from the cotyledons until their subsequent senescence and abscission, were used for the experiments.

Estimations of the PME activity were made on sections of tissue 4–5 cells thick cut from selected parts of the blade, pulvinus, or petiole (Fig. 1).

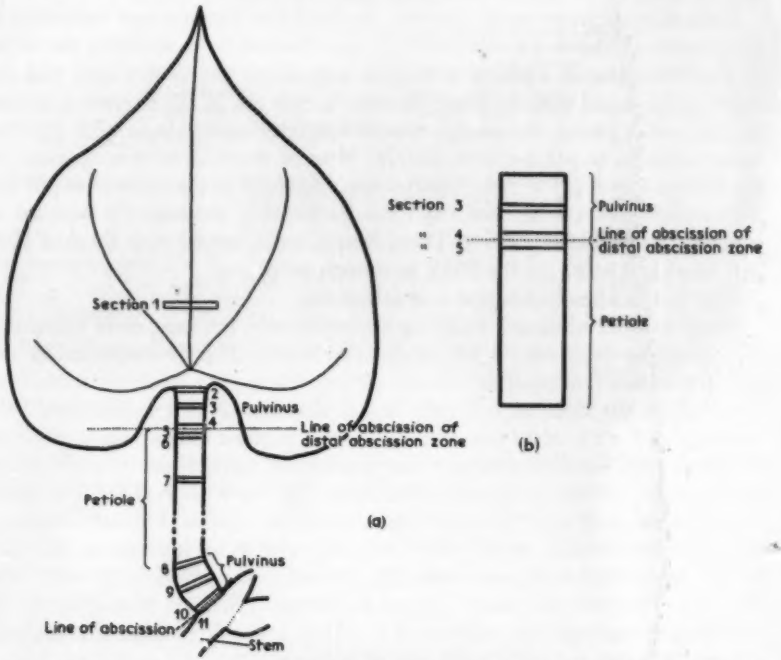

FIG. 1 (*a*). Primary leaf of *Phaseolus vulgaris* showing position of sections assayed for pectin methylesterase activity.
(*b*) Excised abscission zone (explant) showing the position of similar sections.

Sections from either side of the line of abscission in material which had not yet undergone separation were obtained by first making a small incision around the clearly visible junction of pulvinus and petiole (distal zone) or pulvinus and stem (proximal zone); the two parts then separated readily along this line of abscission, and a section was cut off from each exposed surface.

The distribution of the enzyme was determined both in whole plants, and in excised abscission zones (explants). Abscission of the leaves was accelerated by removal of leaf blades or by foliar applications of disodium 3:6-endoxy-hexahydrophthalate (endothal) and retarded by treating the leaves with

n-butyl ester of 2:4:5-trichlorophenoxyacetic acid (2:4:5-T). The excised abscission zones consisted of a section 1 cm. long cut from the stalk of the primary leaf, to include the whole of the distal pulvinus, the adjacent abscission line and part of the petiole below. Explants were aligned on grooved perspex racks which were mounted in Petri dishes over 3 per cent. agar and maintained in darkness at 24·5° C. Abscission was accelerated by treatment with ethylene and retarded by applications of 2:4-dichlorophenoxyacetic acid (2:4-D) to the cut surface of the pulvinar end.

Estimation of pectin methylesterase. Initially the enzyme was extracted in two fractions. The water-soluble PME was obtained by macerating the tissue in distilled water at a pH of 6–7. The suspension was centrifuged and the supernatant liquid used for assay. In order to free any of the enzyme adsorbed on cell-wall material the residue was re-extracted three times with distilled water adjusted to pH 8·0 with NaOH. Most of the activity was obtained in the first extract at pH 6–7, with only a small addition in the extracts at pH 8·0. The addition of 0·1 M. NaCl did not measurably increase the amount of esterase extractable at pH 6–7. These results are in accord with those of Hills and Mottern (1947) for the PME in tomato pulp.

The method finally adopted was as follows:

Forty sections of tissue, weighing approximately 100 mg., were macerated in a grinding tube with 7 ml. of distilled water. The fine suspension was transferred to a Lusteroid tube and centrifuged at 4,500 r.p.m. for 10 minutes. 2·25 ml. of the clear supernatant liquid was added to a colorimeter tube together with 2 ml. of a 1 per cent. solution of purified citrus pectin, made up in 0·1 M. NaCl and containing 0·00175 per cent. methyl red. The pH of the pectin solution was previously adjusted to pH 6·0 with NaOH. The tubes were incubated at 24·5° C. The liberation of free carboxyl groups resulting from de-esterification of the pectin was followed as an increase in intensity of the red colour using an Evans Electro-Selenium colorimeter with filter No. 624. Over the pH range 5·5–5·2 this bears a linear relationship to the colorimeter reading. The addition of 2:4-D or 2:4:5-T to these enzyme/pectin mixtures did not affect their rate of reaction.

All esterase activities are expressed in arbitrary units, namely the change in the colorimeter reading induced by 1 g. of tissue (fresh weight) per 100 min. in a standard solution of pectin under standard conditions. Unless otherwise stated (Table I) duplicate estimations were carried out on each of two duplicate samples of tissue.

The percentage dry weights of the different parts of the leaf blade, pulvinus and petiole (Fig. 1) of primary leaves of various ages were found to be equal; the relative values of the PME activities are not, therefore, appreciably altered if they are expressed on a dry weight basis.

The fresh weight of explants kept at 100 per cent. humidity at 24·5° C. remained virtually unchanged for periods up to 6 days, but the percentage dry weight fell progressively as metabolic substrates became depleted. The percentage dry weight was, however, the same at any one time for all the

sections from any one treatment. Thus a comparison of relative PME activities for the tissues at any one time is valid if the results are expressed on a dry-weight basis, but the values at different times are not strictly comparable on this basis.

TABLE I

The level of PME activity in sections 1–7 from blade, pulvinus, and petiole in leaves of various ages, together with the abscission time for explants cut from these leaves

State of leaf	Days from unfolding of primary leaf	PME activity (arbitrary units) in sections from: Blade 1	Pulvinus: Top 2	Pulvinus: Middle 3	Pulvinus: Abscission zone 4	Petiole: Abscission zone 5	Petiole: Adjacent tissue 6	Petiole: Middle 7	Ratio of activity sections 4:5	Average time for explants to abscind hr.
Just unfolding	0	—	—	—	—	—	—	—	—	39
expanding	5	1·50	5·73	5·64	7·85	3·40	2·76	1·51	2·3	45
,,	9	1·58	4·78	5·30	4·78	1·81	1·99	0·93	2·6	50
expanded	13	0·48	3·83	4·12	4·17	1·31	1·62	0·77	3·2	—
,,	16	0·64	3·72	3·55	3·15	1·06	1·61	0·57	3·1	59
,,	21	—	—	—	—	—	—	—	—	62
Fully mature, becoming paler	26	0·34	3·11	—	2·20	1·18	0·49	0·40	1·9	44
Yellowing	30	0·54	2·78	2·46	1·92	0·72	0·68	0·29	2·7	41
Yellow, abscinding	35	0·48	0·95	0·77	0·79	0·71	0·52	0·41	1·1	12

Estimations of protein nitrogen. In some experiments protein nitrogen was estimated in sections 3, 4, and 5 (see Fig. 1) so that the PME activities of these sections could be interpreted in relation to the levels of protein in the tissues.

Method. 50 mg. samples of tissue were macerated in distilled water and the protein fraction precipitated with trichloroacetic acid as described by Galston and Dalberg (1954). After centrifugation the precipitate was collected and its nitrogen content determined colorimetrically by the micro-Nessler method of Yuen and Pollard (1952–4). Duplicate determinations were done on each of two duplicate samples of tissue.

EXPERIMENTAL RESULTS

Experiments with whole plants

Natural abscission. PME activity was determined in a single sample of sections 1–7 (see Fig. 1) cut from primary leaves at various stages of development. Protein nitrogen determinations were made on sections 3, 4, and 5. The first sections were taken when the leaves had just unfolded and the last set were cut just before abscission when the leaves were wholly yellow and senescent. Explants were excised from the plants on the same days as the PME determinations were made and the average time for their abscission under controlled conditions was recorded. The results are shown in Tables I and II. In young and mature leaves, the level of the enzyme activity is relatively higher in the pulvinus than elsewhere, with a sharp drop across the abscission line to low values in the adjoining petiolar sections. The PME activity of each tissue falls progressively as the leaf becomes older. The greatest

decrease occurs in the pulvinus, so that just prior to abscission the striking difference in activity between the adjacent sections 4 and 5 from the pulvinar and petiolar side of the abscission line is no longer observed. Table II shows that there is no clear correlation between the enzyme activity in a section and its content of protein nitrogen.

TABLE II

Values for protein nitrogen in sections 3, 4, and 5, from the pulvinus and petiole of leaves of various ages

Log. transformation values given in italics

State of leaf	Protein nitrogen as mg./g. fresh weight in sections from: Pulvinus: Middle 3	Pulvinus: Abscission zone 4	Petiole: Abscission zone 5
Just unfolding	2·16 (*1·33*)	2·13 (*1·32*)	2·01 (*1·30*)
Expanding	1·68 (*1·22*)	1·79 (*1·25*)	1·80 (*1·25*)
Fully expanded, mature	1·25 (*1·09*)	1·33 (*1·12*)	0·98 (*0·99*)
Yellow, abscinding	0·82 (*0·91*)	0·87 (*0·93*)	0·62 (*0·79*)

Sig. Diff. on transformed values = 0·04 ($P = 0·05$)

TABLE III

The PME activity (arbitrary units) in sections 10 and 11 from the petiole-stem abscission zone of leaves in which abscission has been accelerated by removal of the leaf blade and part of the petiole

Log. transformation values given in italics

	Position of section taken from abscission zone	PME activity: Number of days for which leaves were debladed: 0	2	3	4*
Experiment 1. Young, fully expanded leaves	10. Pulvinus	3·10 (*1·49*)	1·83 (*1·26*)	—	0·63 (*0·79*)
	11. Stem	1·93 (*1·28*)	1·25 (*1·09*)	—	0·91 (*0·95*)
		Sig. Diff. on transformed values = 0·09 ($P = 0·05$)			
Experiment 2. Older, fully mature leaves	10. Pulvinus	1·47 (*1·16*)	1·35 (*1·13*)	1·23 (*1·08*)	0·54 (*0·73*)
	11. Stem	0·70 (*0·84*)	0·68 (*0·83*)	0·71 (*0·85*)	0·55 (*0·74*)
		Sig. Diff. on transformed values = 0·12 ($P = 0·05$)			
	Ratio of activity sections 10:11				
	Experiment 1	1·60	1·46	—	0·69
	Experiment 2	2·10	1·99	1·76	0·98
			Sig. Diff. = 0·32 ($P = 0·05$)		

* All the petiolar stumps debladed for 4 days had abscinded in both experiments.

Accelerated abscission. (*a*) *Removal of leaf blades.* La Rue (1936) and many later workers have shown that removal of the leaf blade will accelerate separation in the abscission zone below. Experiments were therefore designed to

determine the effect of such a treatment upon the PME activity of adjacent sections (10 and 11) taken from the pulvinar and stem sides of the line of the proximal abscission zone (Fig. 1) of young, though fully expanded, leaves.

The blade and part of the petiole were removed from the leaf stalk leaving a stump some 1·5 cm. long, which included the whole basal pulvinus and a part of the petiole. This procedure was repeated on the 2 following days on other plants sown at the same time. On the 4th day after the start of the experiment, the stumps were abscinding from plants whose leaves had been

TABLE IV

The PME activity (arbitrary units) in sections 3, 4, and 5, from the pulvinus and either side of the abscission line of leaves in which abscission had been accelerated by a foliar application of Endothal at 500 p.p.m.

Log. transformation values given in italics

	PME activity							
	Control leaves			Endothal-treated leaves				
	Pulvinus		Petiole	Pulvinus		Petiole	Ratio of activity sections 4:5	
Time after treatment (days)	Middle 3	Abscission zone 4	Abscission zone 5	Middle 3	Abscission zone 4	Abscission zone 5	Control leaves	Treated leaves
0	3·07 (*1·48*)	2·59 (*1·41*)	0·69 (*0·83*)	—	—	—	3·72	—
2	2·87 (*1·45*)	2·30 (*1·36*)	0·71 (*0·85*)	2·31 (*1·36*)	1·75 (*1·24*)	0·84 (*0·92*)	3·20	2·07
4	2·61 (*1·41*)	2·37 (*1·37*)	0·81 (*0·90*)	1·44 (*1·15*)	1·60 (*1·20*)	0·75 (*0·87*)	2·90	2·13
	Sig. Diff. on transformed values = 0·09 (P = 0·05)						Sig. Diff. = 0·68 (P = 0·05)	

removed on the 1st day. Pulvinar and stem sections were then cut from all the stumps as well as from intact leaf stalks and assayed for PME activity. The data for two separate experiments are shown in Table III. It is evident that the removal of the blades causes a fall in the PME activity of both pulvinar and stem tissue and that both tissues show a similar low activity at the time of abscission.

(*b*) *Chemical defoliation.* Endothal is an effective commercial defoliant and desiccant. An aqueous solution of 500 p.p.m. was applied to the bean leaves wetting the whole upper surface of the blade. A streak of vaseline across the base of the blade prevented contamination of the petiole or pulvinus, or the area of blade adjacent to the pulvinus. Some patches of the blade were desiccated within 24 hours, and by the 4th day the non-desiccated parts were yellowing. Abscission took place between the 4th and 6th day from treatment. Sections 3, 4, and 5 from the middle of the pulvinus and from either side of the pulvinar-petiolar abscission line were cut from treated and control leaves at the start of the experiment and 2 and 4 days after treatment.

The PME activities of these sections are listed in Table IV. The results show that compared with the controls, there is a rapid fall in the PME activity of the pulvinar tissue of the leaves treated with endothal.

Retarded abscission. Abscission may be artificially retarded by applications of synthetic growth regulators to the leaf.

Two drops, each of 0·005 ml., of a 10 per cent. solution of the *n*-butyl ester of 2:4:5-T in 95 per cent. alcohol were applied to the upper surface of the blade. The PME activities were determined after 6 days.

The results of PME determinations in pulvinar and petiolar tissue of leaves so treated are given in Table V.

TABLE V

The PME activity (arbitrary units) in sections 3, 4, and 5, from the pulvinus and either side of the asbcission line of control leaves, and leaves in which abscission has been retarded by a foliar application of 2:4:5-T n-butyl ester. The figures for percentage abscission refer to the explants which were cut from these leaves on the sixth day of the experiment

Log. tranformation values given in italics

Treatment	Duration of treatment (days)	PME activity: Pulvinus, Middle 3	Pulvinus, Abscission zone 4	Petiole, Abscission zone 5	Ratio of activity sections 4:5	% abscission of explants at 120 hrs.
Control	0	2·49 (*1·39*)	2·24 (*1·35*)	0·82 (*0·91*)	2·74	—
,,	6	2·05 (*1·31*)	1·73 (*1·23*)	0·55 (*0·74*)	3·14	80
2:4:5-T. 1·0 mg./leaf	6	3·47 (*1·54*)	2·57 (*1·40*)	0·49 (*0·69*)	5·25	0
		Sig. Diff. on transformed values = 0·09 ($P = 0·05$)			Sig. Diff. = 0·63 ($P = 0·05$)	

The 2:4:5-T treatment resulted in a significantly higher PME activity in the pulvinar tissue compared with the control values, while the figures for the percentage abscission at 120 hours for explants cut from the treated and control leaves show that separation is markedly delayed by the foliar application of the growth regulator.

Experiments with explants

Explants were cut from fully expanded, young primary leaves, and mounted over agar as described earlier. Protein nitrogen determinations and assays of PME activity were made on sections of tissue (3, 4, and 5) from the middle of the pulvinus and either side of the abscission line of control explants, and of those in which abscission had been accelerated or retarded.

Accelerated abscission. Open Petri dishes of mounted explants were stacked on racks beneath large bell jars which were then sealed to glass plates. A small amount of ethylene from a pressure cylinder was introduced into some bell jars to give an unknown, but low, concentration of ethylene in the internal atmosphere.

The bell jars were kept in darkness at 24·5° C. for 24 hours and then opened out of doors. A lid was immediately put on each Petri dish and they were returned to the dark room. At various intervals from the start of the experiment, explants were removed from the dishes and sections of the tissues were analysed. The results are recorded in Table VI.

The controls show a steady fall in PME activity in all sections, abscission

being complete between 48 and 72 hours when there is little difference in activity between the adjacent sides of the abscission line. Ethylene treatment caused a more rapid decrease in activity in the pulvinar section next to the line of abscission, and within 24 hours, this value had fallen below that in the adjacent petiolar section. These explants abscinded between 24 and 41 hours.

TABLE VI

The PME activity (arbitrary units) in sections 3, 4, and 5, from the pulvinus and from either side of the line of abscission of explants in which abscission has been accelerated by the presence of ethylene gas. Values for protein nitrogen in sections of explants similarly treated are given below

Log. transformation values given in italics

	PME activity							
	Control explants (100% abscission between 48 and 72 hrs.)			Ethylene-treated explants (100% abscission between 24 and 41 hrs.)				
	Pulvinus		Petiole	Pulvinus		Petiole	Ratio of activity sections 4:5	
Length of treatment hr.	Middle 3	Abscission zone 4	Abscission zone 5	Middle 3	Abscission zone 4	Abscission zone 5	Control explants	Treated explants
0	2·70 (*1·43*)	2·37 (*1·37*)	1·07 (*1·02*)	—	—	—	2·21	—
24	2·20 (*1·30*)	1·20 (*1·07*)	0·84 (*0·92*)	1·90 (*1·27*)	0·58 (*0·76*)	0·73 (*0·86*)	1·45	0·80
48	1·34 (*1·12*)	0·50 (*0·69*)	0·34 (*0·53*)	1·25 (*1·09*)	0·26 (*0·42*)	0·44 (*0·64*)	1·44	0·59
72	1·41 (*1·14*)	0·49 (*0·69*)	0·35 (*0·54*)	—	—	—	1·40	—
	Sig. Diff. on transformed values = 0·13 ($P = 0·05$)						Sig. Diff. = 0·32 ($P = 0·05$)	
	Protein nitrogen mg./g. fresh weight							
0	1·71 (*1·23*)	1·69 (*1·22*)	1·53 (*1·18*)	—	—	—	—	—
48	1·12 (*1·04*)	1·12 (*1·04*)	0·99 (*0·99*)	1·02 (*1·00*)	0·91 (*0·96*)	1·17 (*1·06*)	—	—
	Sig. Diff. on transformed values = 0·04 ($P = 0·05$)							

Although the protein nitrogen values fall during the course of abscission, the general distribution of PME activity and the rapid decrease that occurs in the pulvinar tissue next to the abscission line in ethylene-treated explants is not reflected in an unequal change in the protein content of the different tissues.

Retarded abscission. Explants from untreated plants were cut and mounted over agar in the usual way. The abscission of some of these explants was retarded by an application to the cut end of the pulvinus of an 0·002 ml. drop of an aqueous solution of the sodium salt of 2:4-D at 20 p.p.m. Other explants, to which an 0·002 ml. drop of distilled water was applied, served as controls. Sections of tissue were analysed for PME activity when the controls had started to abscind (48 hours) and again at 96 hours, when abscission of the controls was complete, but when the 2:4-D-treated explants showed no sign of separation. Results are given in Table VII. The PME activity falls rapidly in control explants, and again, abscission occurs at a time when similar levels of activity are obtained on either side of the abscission line. No fall occurs, however, in the pulvinar tissues (Sections 3 and 4) of the 2:4-D-treated explants, in which abscission is retarded.

The figures for the protein nitrogen of these sections determined at 72 hours from the start of the experiment show a somewhat smaller drop in the pulvinar values for the 2:4-D-treated explants, but there is no apparent correlation between PME activity and protein nitrogen content.

TABLE VII

The PME activity (arbitrary units) in sections 4 and 5 from either side of the abscission line of explants in which abscission has been retarded by applications of 2:4-D. Values for protein nitrogen in sections 3, 4, and 5, of explants similarly treated are given below

Log. transformation values given in italics

	PME activity							
	Control explants (100% abscission by 96 hrs.)			2:4-D-treated explants (no abscission by 96 hrs.)			Ratio of activity sections 4:5	
	Pulvinus		Petiole	Pulvinus		Petiole		
Length of treatment hr.	Middle 3	Abscission zone 4	Abscission zone 5	Middle 3	Abscission zone 4	Abscission zone 5	Control explants	Treated explants
0	—	3·31 (*1·52*)	1·67 (*1·22*)	—	—	—	1·98	—
48	—	1·33 (*1·12*)	1·33 (*1·18*)	—	2·34 (*1·52*)	1·25 (*1·09*)	0·87	2·67
96	—	0·76 (*0·88*)	1·11 (*1·04*)	—	3·84 (*1·58*)	1·42 (*1·15*)	0·69	2·70
	Sig. Diff. on transformed values = 0·08 ($P = 0·05$)						Sig. Diff. = 0·33 ($P = 0·05$)	
	Protein nitrogen mg./g. fresh weight							
0	1·72 (*1·23*)	1·89 (*1·27*)	1·61 (*1·20*)	—	—	—	—	—
72	0·71 (*0·85*)	0·74 (*·086*)	0·96 (*0·98*)	0·98 (*0·99*)	0·99 (*0·99*)	0·93 (*0·96*)	—	—
	Sig. Diff. on transformed values = 0·06 ($P = 0·05$)							

DISCUSSION

The results have shown that in all but abscinding leaves the PME activity in the pulvinus tissue is higher than in the other parts of the blade or petiole. The level of enzyme activity is relatively high in very young leaves, but falls progressively in each tissue as the leaf matures, the largest fall occurring in the pulvinus.

There appears to be no direct correlation between the actual level of enzyme activity and the rapidity of abscission (Table I). Explants which are cut from very young leaves, high in PME, abscind quickly, but as the leaf grows older the enzyme activity falls steadily while the abscission time first increases to a maximum value and later decreases again as the leaves become senescent. However, a consideration of these results suggests that the steepness of the gradient of PME activity across the separation line (ratio of activity of sections 4:5) may be linked to the time required for abscission. The gradient increases until the leaf is fully expanded, and is highest when the values for the abscission time are maximal. Both gradient and abscission time fall again as the leaf becomes senescent, and at the time of leaf fall when the level of PME is low in all the tissues, the gradient across the abscission line ceases to exist and may eventually be reversed.

The data in other tables show a similar trend. Where abscission is

accelerated, the decrease in the level of PME in the pulvinus is more marked than in the controls, so that the gradient across the abscission line falls more rapidly to the low values associated with separation (Tables III, IV, and VI). When abscission is delayed by a suitable application of a growth regulator (Tables V and VII), the natural drop in PME activity is retarded and the gradient of activity from the pulvinus across the abscission line is maintained, or increased. There does not appear to be any direct relationship between the protein nitrogen values in the sections and the observed PME activities.

Several reports of increases in PME activity following applications of growth regulators have appeared in the literature. Bryan and Newcomb (1954) claim increases in cultured tobacco pith tissue supplied with indole-3-acetic acid (IAA), Waygood *et al.* (1954) for extra-cellular PME by *Cladosporium herbarum* grown in a medium incorporating 2:4-D or 2:4:5-T, and Neely *et al.* (1950) for bean plants treated with 2:4-D. In the latter case, however, the enzyme activity was not determined prior to treatment and it is possible that 2:4-D was effective only in retarding the natural decrease in PME activity that occurs in control plants.

PME is a specific enzyme, normally associated with the hydrolysis of pectic methyl esters, but its role in plant metabolism is debatable. The relation of the PME changes in the abscission zone to the actual separation processes is in no way clear, but it is possible that the high PME activity on the pulvinar side of the intact abscission line ensures a low content of soluble esterified pectin and a relatively high content of insoluble pectic wall material with few ester groups. In this way the adhesion of the cell walls along the line of abscission may be maintained. When the PME activity on both sides of the abscission line is low, the soluble more highly esterified pectins might then increase at the expense of the insoluble middle lamella and cell-wall materials so that separation readily takes place. Sampson (1918), using staining techniques, has shown that cells in the abscission zone of *Coleus* are low in calcium at the time of abscission and his evidence points to an increase in soluble pectin at separation. Increases in soluble esterified pectin are known to occur during ripening and senescence in fruit with a corresponding decrease in insoluble cell wall and middle lamella pectins of low ester content (Fisher, 1943, for apples). Some of the changes that take place in the cells along the line of abscission may well be similar to those occurring in ripening fruits. Both ripening and abscission are accelerated in the presence of ethylene, and Heid (1941) has shown that the treatment of orange peel with ethylene induces a more rapid disappearance of insoluble pectic compounds. Yagar and Muir (1958) have recently reported that abscission of tobacco ovaries is accelerated by treatment with methionine which might be due to an increase in the methyl ester content of pectin in the abscission zone.

In his studies of pectic enzymes in pears, Weurman (1954) has found that the PME activity is high in the growing fruit, but decreases with age and reaches a low value at ripeness. His data, and the present work with abscission zones, suggest that high PME activity may be associated with a relatively

low content of soluble esterified pectin and a high level of insoluble pectic materials.

Glasziou (1957) states that, *in vitro*, IAA and 2:4-D increase the binding of PME to the cell walls, and has shown (1958) that there is a decrease in the amount of enzyme passing out of the cells of tobacco pith placed in pectin solutions containing 2:4-D. If the adsorbed enzyme were inactive a *decrease* in the rate of de-esterification of pectins might be expected in tissues treated with IAA or 2:4-D. He suggested that his data might support the proposal of Bennet-Clark (1956) that cell walls in which the pectins were esterified (e.g. those treated with auxin) would enlarge more readily by plastic extension than cells in which the pectic materials are present as free acids or as salts, but there is no clear evidence that PME is inactivated when it is adsorbed to cell walls *in vivo*.

Ordin, Cleland, and Bonner (1957), however, have demonstrated that the incorporation of the carbon of C^{14}-methyl-labelled methionine into the pectins in the cell walls of *Avena* coleoptiles is increased in the presence of IAA, which suggests that an increased methyl esterification of the carboxyl groups of the pectin may be associated with auxin-controlled cell extension.

It is therefore of interest that the present results with abscission zones indicate that auxins can maintain or increase above that in non-auxin-treated tissues the measurable activity of the enzyme (PME), believed to be responsible for the de-esterification of pectins.

ACKNOWLEDGEMENTS

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Studies on Plant-Growth Hormones

VI. THE NATURE OF INHIBITOR-β IN POTATO

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SUMMARY

1. The chemical nature of a plant growth inhibitor in potato tuber peel, the 'Inhibitor-β' which commonly occurs on chromatograms of many plant extracts, has been examin

2. The inhibitic ., as indicated by bioassays using wheat coleoptile sections, could not be associated with any particular compound, but was partly or entirely due to a complex mixture of aliphatic acids.

3. Azelaic acid and the coumarin, scopoletin, were isolated together with a new substance, Acid A; degradative evidence is not sufficient to enable a complete structure to be proposed for this acid, but it appears to be an unsaturated polyhydroxy fatty acid.

4. The growth of coleoptile sections in solutions of β at several concentrations was examined over the first 7 hours of growth. Inhibition did not occur until 4 hours; visible damage to the cells of the tissues appeared after this period. When β was examined in a mixture with 3-indolylacetic acid, inhibition was evident after 1 hour. These results are interpreted and the chemical system in which β may operate in growth is briefly considered.

INTRODUCTION

FOR more than three decades it has been known that inhibitors of plant growth may occur in extracts of various plant materials but the literature on their occurrence does not appear to have been reviewed in a comprehensive manner; however, certain aspects of plant development have been considered (e.g. germination inhibitors, Evenari, 1949). When these substances occur in the tissues in sufficiently high concentrations it has sometimes been possible to identify them chemically, but the chemical nature of many inhibitors reported in the literature is not known. The opinion has gradually formed that some of these inhibitors may have a physiological role in growth and that they may participate in the regulation of plant development with the naturally occurring hormones. Extensive work related to correlative growth supporting this concept has been published recently by Libbert (1954 *a* and *b*, 1955 *a*, *b*, and *c*, and 1957) while other workers have related inhibitors to a possible cause of dormancy in seeds (e.g. Wareing and Foda, 1956; Black, 1956; and Flemion, 1956) and in potato tubers (Hemberg 1942–54, this literature is summarized by Burton, 1956).

The work of Bennet-Clark and Kefford (1953), who separated by paper chromatography growth-promoting and inhibiting acidic ether-soluble substances in plant extracts, showed by *Avena* coleoptile section bioassay that

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an inhibitor (termed 'β') was usually present on ammoniacal *iso*propanol-developed chromatograms at approximately Rf 0·6. This inhibitor occurred in extracts of a wide range of materials including seedling shoots, roots, and rhizomes, and in potato tubers. Kefford (1955 *a* and *b*) has examined the distribution of β and other growth regulators in various parts of young bean seedling shoots with a view to explaining the phenomenon of apical dominance in terms of the opposing actions of promoters and inhibitors and has examined interactions of β with various promoters, while Bennet-Clark and Kefford (1954) have contemplated the treatment of these interactions by the auxin kinetics of Bonner and Foster (1955).

As β is widely occurring, and in view of the possible physiological significance of this substance(s) in regulating plant growth, it was decided to examine its chemical nature in order to facilitate future studies of the above. 'Skin' of potato tubers was chosen as an experimental material on the advice of Professor T. A. Bennet-Clark who recommended it as a rich source of β (see Kefford, 1955*a*).

EXPERIMENTAL

Melting-points were determined on a Kofler block and are corrected. Ether extracts were dried over anhydrous magnesium sulphate before evaporation.

Bioassay techniques. Test solutions were examined for inhibitor activity using coleoptile sections of wheat (var. *Atle*). The techniques have been described in detail elsewhere (Bentley and Housley, 1954); briefly, they are as follows. Seeds are germinated and grown in a coarse sand at 25° C. and 90 per cent. relative humidity in the presence of phototropically inactive light as a matter of routine. Coleoptiles of approximately uniform length (usually 1·8–2·0 or 2·0–2·2 cm. in the present work) are selected for use after 62–65 hours and a 10 mm. section is cut 3 mm. from the tip of each one. Mean elongations during 17 hours of 10 sections are given; water controls are mean elongations of 30 sections. After elongation, sections are measured with the aid of a photographic enlarger (×4 magnification), the lengths of the projected images being determined to the nearest millimetre with a flexible rule. For growth curves, phototropically inactive light is used in the enlarger.

Paper chromatography. Descending chromatography on Whatman No. 1 paper was carried out in the dark with *n*-butanol saturated with 1·5N. ammonia until the solvent front was 25–30 cm. from the starting-line. The papers were dried at room temperature in a draught of air; some were examined for fluorescence in ultra-violet light (2537 Å.).

Preparation for bioassay. The paper was divided transversely into 10 equal or unequal strips according to fineness of resolution desired of any particular zone. Each strip was cut into fine segments, placed in 6 ml. ethanol, allowed to stand for 12 or more hours, the ethanol decanted off, and the paper allowed to stand for several hours more in a mixture of 6 ml. water plus 5 ml. ether. The ethanol extract of each strip was evaporated under reduced pressure at a bath temperature of 45° C. and the residue combined with the corresponding

water-ether extract. Concentration of this to approximately 3 ml. removed all organic solvents. The aqueous solutions were filtered and made up to 5 ml. with water. $\times 10$ dilutions, normally tested concurrently, were made using 0·5 ml. for dilution.

Reagents for spraying. (i) Ehrlich's reagent. 2 g. of *p*-dimethylaminobenzaldehyde in a mixture of 20 ml. HCl (S.G. 1·18) and 80 ml. ethanol. (ii) Nitrous-nitric acid. 1 g. KNO_2 dissolved in 20 ml. HNO_3 (S.G. 1·42) was diluted to 200 ml. (iii) Salkowski reagent. A mixture of 1 ml. 0·5M. $FeCl_3$ plus 50 ml. 35 per cent. (w/v) $HClO_4$.

MATERIAL AND ITS EXTRACTION

Several preliminary small-scale extractions and experiments were carried out to find conditions suitable for the large-scale industrial extraction described below.

Skins of potatoes (variety *Majestic*) which had been harvested and clamped in October 1953 and removed for use in April 1954 were collected from the University Refectory kitchens over a period of 15 days. The material was a wet mush produced by a mechanical peeling machine, and unavoidably contained chips of tuber of varying sizes. The mush (1,400 Kg.) was centrifuged until 'dry' (460 Kg.), and extracted in two equal portions with 91 per cent. ethanol (280 Kg.) in a 450 l. vat for 48 hours at room temperature. The alcoholic mushes were combined, gravity-filtered on a nutsche dressed with printer's cloth, and the liquors collected. The nutsche cakes were centrifuged dry (in 40 portions), each portion washed with alcohol (4·5 Kg.) and recentrifuged dry. The total liquors from filtration and centrifuging (730 Kg.) were filtered through double calico cloth and then subjected in portions to a first concentration in a 180 l. mild steel still to a kettle temperature of 54–58° C. at a pressure of 26 mm. mercury. Finally, the combined concentrates (190 Kg.) were further concentrated in the same still (45° C. at 20 mm. mercury) to one-tenth of the weight.

The final concentrate (18 l.), which was largely aqueous, was exhaustively extracted in three portions with ether (5×26 l.). The combined ether extracts were dried, and evaporated to dryness under reduced pressure to remove traces of ethanol. The dark-brown oily residue (48·7 g.) was taken up again in ether (600 ml.) and filtered, and the filtrate extracted with 5 per cent. sodium bicarbonate solution (6×500 ml.). The combined bicarbonate extracts were washed with ether (2×200 ml.), and the aqueous solution acidified at 0° C. with sulphuric acid to pH 3 and exhaustively extracted with ether (5×800 ml.). The combined ether extracts were washed with water (200 ml.), dried, and the ether distilled off under reduced pressure. The yield of acid fraction, a pungent, clear golden-brown oil, was 14·2 g. ($2{\cdot}8 \times 10^{-3}$ per cent. on centrifuged weight). The bioassay histogram of this fraction is shown in Fig. 1.

Preliminary separation of substances in the acid fraction. (i) The acid fraction in chloroform (100 ml.) was left at −40° C. for several days and the crystalline

material which had separated was collected by filtration at 0° C.; recrystallization from aqueous acetone yielded colourless blades (110 mg., $2{\cdot}2\times10^{-5}$ per cent.), m.p. 81° C. The chemical nature of this compound, acid *A*, is discussed on p. 465. (ii) The recovered acid fraction was shaken with benzene (60 ml.) at 50° C. On cooling, some material separated out of solution; the clear benzene layer was then decanted from the syrup and the process repeated twice. The benzene-soluble material amounted to 6·4 g. and the benzene-insoluble residue to 5·1 g.

TABLE I

Weight of material extracted with ether at various pH values from the benzene-soluble and benzene-insoluble fractions of the acidic substances in an extract of potato tuber skins

	Weight of fraction (g.)			
	benzene-soluble		*benzene-insoluble*	
pH				
8·0	BS1	0·46	IB1	0·19
7·0	BS2	0·32	IB2	0·35
6·3	BS3	1·12	IB3	0·58
5·5	BS4	2·24	IB4	2·94
4·2	BS5	1·91	IB5	0·75
2·0	BS6	0·54	IB6	0·75

TABLE II

Final length of coleoptile sections in solutions prepared from the various fractions shown in Table I. 3 mg. of each fraction were dissolved in 5 ml. water. Coleoptile lengths in 3-indolylacetic acid were as follows: 1·0 mg./l., 21·2 mm.; 0·1 mg./l., 18·8 mm.; and 0·01 mg./l., 18·0 mm.

	Coleoptile Length (mm.)			Coleoptile Length (mm.)	
Fraction	*Original Solution*	*× 10 Dilution*	*Fraction*	*Original Solution*	*× 10 Dilution*
H_2O	16·8	16·8	H_2O	16·8	16·8
BS1	14·8	17·3	IB1	14·1	16·2
BS2	10·6	16·9	IB2	12·5	16·7
BS3	11·8	17·7	IB3	10·2	16·4
BS4	13·4	17·1	IB4	10·5	16·7
BS5	10·9	16·4	IB5	12·6	16·9
BS6	11·9	16·6	IB6	11·7	16·1

The benzene-soluble and insoluble fractions were separately dissolved in 3 per cent. sodium bicarbonate solutions (80 ml.) and each solution exhaustively extracted with ether at decreasing pH values. The amounts of material extracted are shown in Table I and bioassay of these fractions in Table II.

Azelaic acid. Fraction BS4 of Table I crystallized on treatment with chloroform; recrystallization from chloroform yielded colourless micro-prisms (322 mg., 7×10^{-5} per cent.), m.p. 107–8° C., undepressed on admixture with an authentic specimen of azelaic acid, m.p. 108° C. (Found: C, 57·4; H, 8·6; E.W., 91·0. Calc. for $C_9H_{16}O_4$: C, 57·5; H, 8·5 per cent.; E.W. 94·1.)

Di-p-bromophenacyl ester leaflets from aqueous ethanol, m.p. 133–4° C.,

undepressed on admixture with an authentic specimen prepared from azelaic acid (Found: C, 51·6; H, 4·6. Calc. for $C_{25}H_{28}O_6Br_2$: C, 51·6; H, 4·5 per cent.).

Di-p-phenylphenacyl ester needles from acetone, m.p. 148° C., undepressed on admixture with an authentic specimen (Found: C, 77·2; H, 6·7. Calc. for $C_{37}H_{36}O_6$: C, 77·0; H, 6·3 per cent.).

Inhibitor isolation. The acid fraction (9·32 g.) was chromatographed in two equal portions on a cellulose column (5·5 × 37 cm.) (column 1) with *n*-butanol equilibrated with 1·5 N. ammonia as the mobile phase. Progress of the column was followed by examination in ultra-violet light; four broad fractions were collected as follows: fraction I (300 ml.), the non-fluorescent forerun together with the first faint-blue fluorescent band (zone 3 in Fig. 1); fraction II (350 ml.), an intensely blue fluorescent band (zone 2 in Fig. 1); fraction III (1·7 l.), tail; and fraction IV (2 l.), ethanol wash. The corresponding fractions from the two columns were combined; the total amount of material in each fraction was: I, 4·45 g.; II, 2·67 g.; III, 1·50 g.; IV, 0·58 g.; a recovery of 98 per cent.

Fraction I was rechromatographed under the same conditions (column II); after a forerun of 330 ml., elution of material commenced and twenty 30 ml. fractions were collected, the number of fractions being limited by bioassay facilities. Fig. 3 shows the bioassay activity of each fraction. Fractions 10–14 were combined (1·56 g.) for further investigation; the bioassay-histogram of this material is shown in Fig. 2 (Found: C, 62·7; H, 9·7: E.W., 223. $C_{12}H_{22}O_4$ requires C, 62·6; H, 9·6 per cent.: E.W., 230). Distillation yielded fraction (*a*) (0·31 g.), b.p. 171–80° C./0·02 mm. (Found: C, 62·9; H, 9·1. $C_{12}H_{20}O_4$ requires C, 63·2; H, 8·8 per cent.) and fraction (*b*) (0·22 g.), b.p. 223–30° C./0·02 mm. (Found: C, 62·5; H, 9·3. $C_{12}H_{22}O_4$ requires C, 62·6; H, 9·5 per cent.).

Fractions 5–9 from column II were combined (1·21 g.) and chromatographed in ether on a column of calcium sulphate (2·3 × 14 cm.). After the forerun, four 5 ml. fractions were collected as the main brown-coloured band was eluted, and then three 30 ml. fractions were collected. Finally, strongly adsorbed material was eluted with ethanol; this fraction (257 mg.), a dark gum, deposited crystalline material from ethyl acetate solution. Recrystallization of this from ethyl acetate gave acid *C* (3·5 mg.), m.p. 84–85° C. (Found: C, 64·7; H, 10·5; E.W., 258. $C_{14}H_{28}O_4$ requires C, 64·6, H, 10·8 per cent.; E.W., 260). Fractions (*a*), (*b*), and (*c*) were partly crystalline and acid *A* (26·4 mg.) was separated from the non-crystallizable syrup. The other fractions were recombined and rechromatographed, in ether, on a calcium sulphate column (2·3 × 16 cm.). This effected little real separation of the crystalline components, but rather separated them from the large amount of non-crystallizable oil which was eluted from the column more quickly. The crystalline material obtained was freed from contaminating syrup by washing with ether at 0° C., and was then fractionally crystallized. The more soluble fraction in chloroform was purified as acid *F* (6·7 mg.), m.p. 91–92° C. (Found: C, 63·0; H, 10·2; E.W., 252. $C_{13}H_{24}O_4$ requires C, 63·7; H, 9·9 per

cent; E.W., 244). The less soluble material was recrystallized from acetone-light petroleum (2:1) to give as the less-soluble fraction, acid *E* (2·3 mg.), m.p. 120° C. (Found: C, 66·0; H, 11·9. $C_{12}H_{24}O_3$ requires C, 66·7; H, 11·1 per cent.). The mother liquors contained acid *D* (3·9 mg.), m.p. 100–1° C. (Found: C, 65·2; H, 10·0; E.W., 262. $C_{14}H_{26}O_4$ requires C, 65·1; H, 10·2 per cent.; E.W., 258). The residual dark syrup was distilled to give a fraction (0·39 g.), b.p. 170–5° C./0·02 mm. (Found: C, 64·5; H, 10·0. $C_{10}H_{18}O_3$ requires C, 64·6; H, 9·7 per cent.); the higher boiling residue was then treated with diazomethane and the methyl esters distilled to give a fraction (0·19 g.) b.p. 200–10° C./0·02 mm. (Found: C, 68·8; H, 9·8. $C_{13}H_{22}O_3$ requires C, 69·0; H, 9·8 per cent.).

Isolation of scopoletin. Fraction II from the cellulose column I was chromatographed on alumina (50 g.) in darkness. After development with benzene (1 l.) the band which fluoresced intensely blue in ultra-violet light was eluted with ether. The material obtained on evaporation of the ether was recrystallized from benzene as rectangular needles (0·45 mg.), m.p. 195–8° C. with prior sublimation. Light absorption in ethanol: Max. 2290, 2540, 2960, and 3450 Å.; log ϵ 4·1, 3·65, 3·69, and 4·06 respectively. Andreae (1948) reports m.p. 206° C. with sublimation, and light absorption: Max. 2290, 2540, 2980, and 3460 Å.; log ϵ 4·2, 3·72, 3·77, and 4·12 respectively. When chromatographed on paper in three separate solvent systems (*n*-butanol saturated with 1·5 N. ammonia; *n*-butanol—water—acetic acid: 4–5–1; and *iso*propanol—water: 1–4) with a sample of authentic scopoletin, the natural product chromatographed with the authentic compound in a similar manner giving identical Rf values (0·27, 0·82, and 0·50 respectively). It was not possible to isolate the other fluorescent compounds present in lower concentrations in fraction II.

RESULTS AND DISCUSSION

Chemical. The potatoes used had been commercially supplied to the University Refectory kitchens. Preliminary work confirmed the presence of acidic ether-soluble growth-inhibiting substances in the peel of this material. Bioassays of extracts of paper chromatograms indicated a distribution of inhibiting material on the papers similar to that previously reported by Bennet-Clark and Kefford (1953) and Kefford (1955*a*). At first, the extractions were made at 0° C. and the extracts then concentrated under reduced pressure at a temperature below 30° C., but having regard for the facilities which would be available for an industrial extraction, it was found that the isolation of the inhibitor material was unimpaired by extraction with 91 per cent. ethanol at room temperature and distillation of the solvent under partially reduced pressure at a temperature below 60° C. The large-scale extraction of potato peel (460 Kg.) yielded 14·2 g. syrup which was worked up for the ether-soluble acid fraction.

Examination of this material was guided by the results of bioassays using wheat coleoptile sections; a histogram is shown in Fig. 1. Some growth promotion occurred at the position of 3-indolylacetic acid (IAA) (Rf 0·19–0·28)

on a × 10 dilution but none of the characteristic colour reactions of IAA could be detected when the chromatogram was sprayed with ferric chloride-perchloric acid, nitrous-nitric acids, and *p*-dimethylaminobenzaldehyde. The main zone of inhibition occurred at Rf 0·4–0·8, which corresponds with the position of inhibitor-β in the *n*-butanol/ammonia solvent system. This

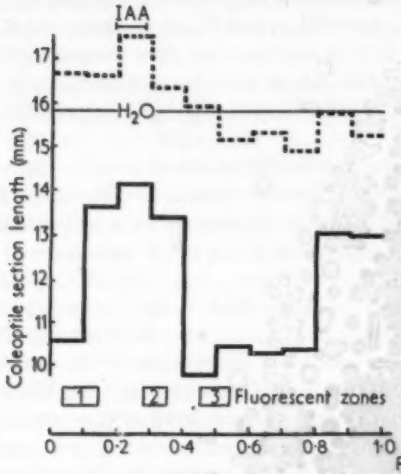

FIG. 1

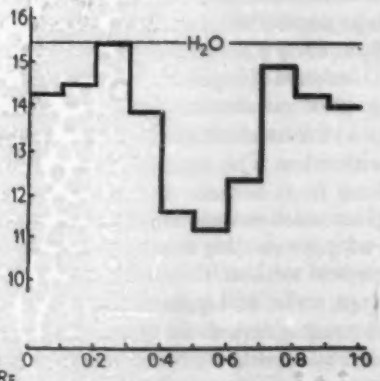

FIG. 2

FIG. 1. Distribution of growth inhibitors and promoters on a *n*-butanol/ammonia-developed chromatogram of the acidic substances in an extract of potato tuber peel. A × 10 dilution is indicated by broken lines. 60 mg. of material were chromatographed on a 35 cm. paper. A marker spot of 3-indolylacetic acid (IAA) and the following zones of fluorescence in ultra-violet light are shown: zone 1, bluish-purple; zone 2, ice blue; zone 3, weak blue.

FIG. 2. Distribution of growth inhibitors on a *n*-butanol/ammonia-developed chromatogram of the concentrated inhibitor material (fractions 10–14 off column II). 60 mg. of material were chromatographed on a 20 cm. paper.

zone also gave no colour reaction; bromothymol blue indicator for acids gave a continuous streak between Rf 0·35 and 0·78, which suggested the complexity of the mixture subsequently found. None of the extracts of sections of paper chromatograms possessed a characteristic ultra-violet light absorption spectrum, apart from end-absorption; indeed, the only means available for detecting the zone of inhibition was by bioassay. It was, however, possible to make use of trace compounds present in the acid fraction with strong fluorescence in ultra-violet light, since they produced a readily detectable pattern on chromatograms as indicated in Fig. 1. It is seen that the zone of inhibition lies just ahead of the weakly fluorescent zone 3, and ahead of the intensely fluorescent zone 2. Since these substances chromatographed on columns as well-defined bands readily visible in ultra-violet light, they served as valuable reference points for following the elution of the inhibitor band.

The total acid fraction was first subjected to treatments to separate as much

crystalline material as possible. A chloroform solution of the syrup yielded on standing at −40° C. a homogeneous crystalline compound, acid *A* (110 mg.); available chemical evidence (reported in detail by Taylor, 1956) suggests a structure of the type $R.CH{:}CH.(CHOH)_4.(CH_2)_7.COOH$ where R is possibly a C_4 alkyl group. No other crystalline material could be obtained in this manner. Next, the syrup was extracted with benzene; the soluble and insoluble fractions were taken up separately into sodium bicarbonate solution

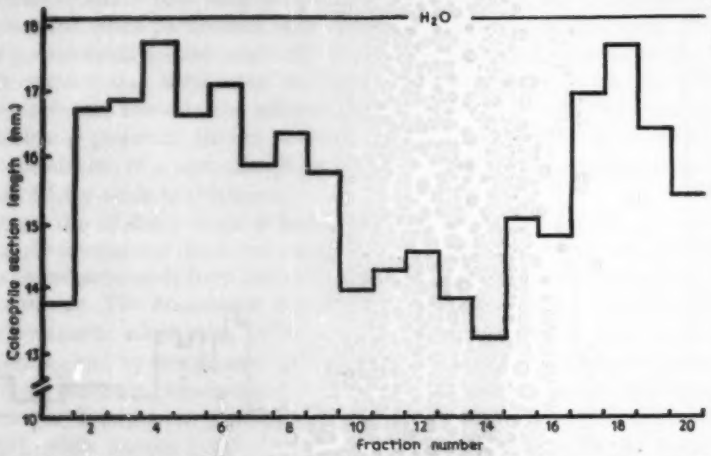

FIG. 3. Distribution of inhibitor activity in eluates obtained by chromatography on cellulose (column II) of acidic substances in an extract of potato tuber peel. Each eluate was assayed at a concentration of 600 mg./l.

and each extracted with ether at decreasing pH values. At pH 4·2 a crystalline acid (350 mg.) was extracted from the benzene-soluble fraction. This acid was identified as azelaic acid (heptane-1:7-dicarboxylic acid) by melting-point and mixed melting-point, analysis, equivalent weight, and preparation of the di-*p*-bromophenacyl and di-*p*-phenylphenacyl esters, see p. 462. Azelaic acid has been previously isolated from urine (Lederer and Polonsky, 1948) but the present isolation appears to be the first from plant tissues. Neither acid *A* nor azelaic acid caused inhibition at a concentration of 50 mg./l. No other crystalline material could be separated conveniently in this manner. Bioassays indicated that little real concentration of inhibitor had occurred into any of the above fractions.

The combined material was then submitted to column partition chromatography on cellulose using *n*-butanol/1·5 N.-ammonia as the solvent system (column I). An initial separation was achieved by collecting the material eluted ahead of the band corresponding with zone 2. This was then rechromatographed (column II) and the eluates fractionally collected. Fig. 3 shows that the inhibition was most pronounced in fractions 10–14, and these were combined for further examination. That this material corresponded with the

zone of inhibition originally observed with the crude acid fractions is shown by Fig. 2.

Further concentration of the inhibition activity of this fraction was not achieved by partition chromatography on silica gel using *n*-butanol/ammonia or chloroform/phosphate buffer solvent systems or by adsorption chromatography on calcium sulphate. The inhibition activity at 500 mg./l. was unaffected by prior refluxing for 1 hour with water, N. sulphuric acid, or N.

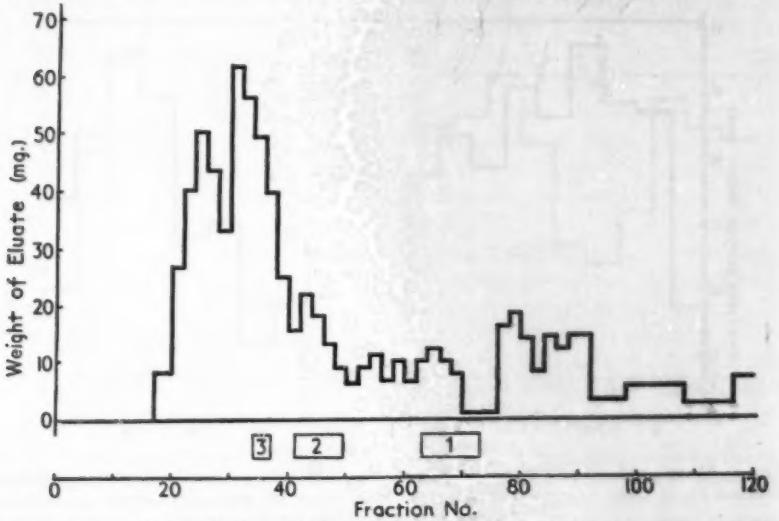

FIG. 4. The weight distribution of acidic substances in an extract of potato tuber peel when eluted from a cellulose column with *n*-butanol/1·5 N.-ammonia. 600 mg. acid fraction were chromatographed on a 4·5 × 15 cm. column. A total of 120 fractions of approximately 2·3 ml. each were collected; they were combined in pairs and the solvent removed by distillation under reduced pressure. The position of fluorescent zones is shown by rectangles labelled 1, 2 and 3.

sodium hydroxide solution, and by treatment with alkaline hydrogen peroxide at 25° C. for 3 hours; the inhibition was completely lost in each case on diluting to 50 mg./l. concentration.

The material (1·56 g.) was a dark syrup from which no crystalline material could be separated by the above chromatographic techniques. While no crystalline carboxylic acid derivatives could be prepared, elementary analyses suggest that a mixture of hydroxymonocarboxylic acids of medium chain length was being handled. Distillation yielded two main fractions, (i) $C_{12}H_{20}O_4$, b.p. 171–80° C./0·02 mm., and (ii) $C_{12}H_{22}O_4$, b.p. 223–38° C./0·02 mm.

Further chemical purification of these fractions was not attempted and the inhibitor examination was closed. This termination was influenced by results about to be described which suggested that much of the observed inhibition of cell elongation results from toxicity, particularly at the higher inhibitor concentrations. In this respect it must be pointed out that in much of the

published work dealing with growth inhibitors the results are purely qualitative observations obtained with total extracts of small quantities of plant tissue without critical purification and without quantitative knowledge of the factors concerned. While growth promotion is a specific property of a limited range of compounds, inhibition of growth can be caused by many types of compounds acting at a sufficiently high concentration. This applies particularly to paper chromatography results in which no attempt is usually made to determine the actual weight-distribution of material on the paper. Fig. 4 shows that when partitioned on a cellulose column the greater proportion of the potato acids moved relatively quickly, ahead of the zone 2 fluorescence; this pattern was duplicated on paper chromatograms, with the greatest concentration towards the solvent front, in the zone with Rf 0·5–0·8—the inhibitor-β position. In the absence of further evidence, it would appear that inhibition of wheat coleoptiles can be ascribed to a fortuitous concentration of fatty acids in this zone.

After the inhibitor fraction had been collected for the preceding work, the strongly fluorescing band corresponding with zone 2 on paper chromatograms was eluted separately from column I and the material further chromatographed on alumina. The fluorescent compound isolated (0·45 mg.) was identified as the coumarin, scopoletin, by melting-point, its ultra-violet light absorption spectrum, and by comparison of Rf values with an authentic scopoletin sample in three different chromatographic solvent systems. Scopoletin has been isolated previously from potato tubers infected with leafroll virus (Andreae, 1948), while Burton (1956) has provisionally identified it by Rf value and chromogenic spray. The more slowly moving material on column I presumably contained, amongst others, acids of the respiration cycle, but the isolation of these was not undertaken.

Bioassays. During routine assays of inhibitor chromatograms lasting 17 or more hours, it was frequently noticed that coleoptile sections in the inhibitory portion of chromatograms showed signs of toxicity involving loss of turgidity, distortion, and a brownish discoloration of the sections. In order to discover when the above symptoms first appeared, the course of section growth through time was followed by measuring length every hour over 1–7 hours. Three concentrations of inhibitor (fractions 10–14 off column II) were used at levels shown in Fig. 5B. Over the first 4 hours, section growth in inhibitor was very similar to that in water, but after this period growth-rates in inhibitor were reduced, particularly at the two higher concentrations. With the latter the first symptom of toxicity appeared after 4 hours; this consisted of loss of turgidity in cells near the cut ends. After approximately 24 hours, sections in 600 mg./l. inhibitor were not fully turgid but section shrinkage had not occurred; however, shrinkage was common in many experiments and an example is given in Fig. 5B (broken-line curve).

When inhibitor was examined, not against endogenous growth of sections in water, but against growth induced by applied IAA, rather different results were obtained (Fig. 5A). Section growth in the presence of IAA+inhibitor

did not follow the course of growth in IAA alone, but diverged from it within the first hour. A similar result was obtained when 0·1 mg./l. IAA was used except that the inhibitor (600 mg./l.)+IAA curve deviated less markedly from the IAA curve over the first 2 hours. With 0·03 mg./l. IAA the two curves

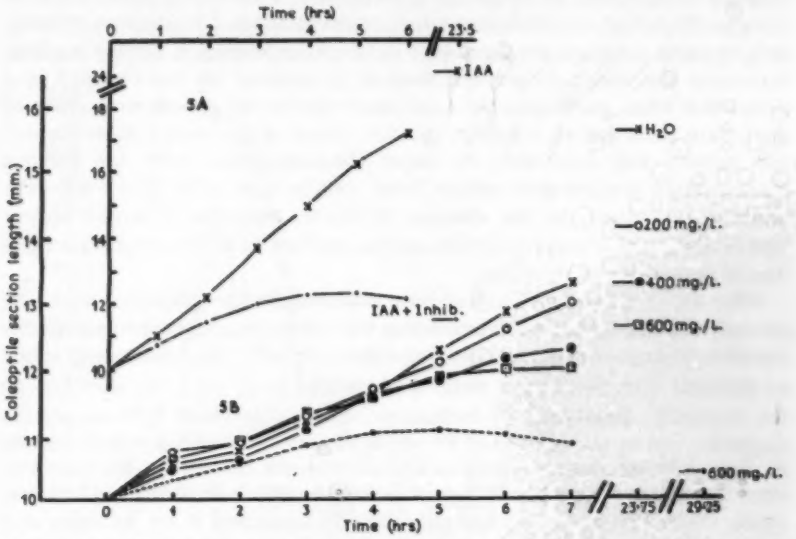

FIG. 5A. Growth of wheat coleoptile sections in 1 mg./l. 3-indolylacetic acid (IAA)+1 per cent. sucrose (—×—), and in 600 mg./l. inhibitor+1 mg./l. IAA+1 per cent. sucrose (—•—).
FIG. 5B. Section growth in water and in solutions of inhibitor at three concentrations. The curve shown by a broken line is a separate experiment.

were virtually superimposed over this two-hour period, while with 0·02 mg./l. IAA and lower concentrations they were superimposed for 3 hours before deviating.

From Fig. 5B it is evident that the inhibitory material from column II did not immediately reduce growth of sections below that in water, and that reduction started only after 4 hours. If one assumes that growth of sections in water is regulated by content of natural auxins, then from the above data one may argue that no substance was present at a high enough concentration which could compete with the auxins for growth sites in the cells, or could quickly block any process vital to the growth reactions. On the other hand, it may be argued that penetration of inhibitory material was slow and that 4 hours elapsed before the material could become effective. At the higher concentrations of inhibitor, inhibition was brought about by a destruction of systems essential for growth including the water permeability-regulating mechanism of the cells. With respect to the lower inhibitor concentrations it has been pointed out by Housley, Bentley, and Bickle (1954) and Barlow, Hancock, and Lacey (1957) that it is not essential for external symptoms of

toxicity to be shown in a tissue before the effects of such toxicity can be exerted on the growth systems present. As symptoms of toxicity appeared at 4 hours at the same time as inhibition commenced, it would be difficult to characterize any inhibition resulting from competition between inhibitor and auxins for growth sites under these conditions.

In the applied-IAA system of Fig. 5A, one may suggest that inhibitor was interfering in uptake of IAA, particularly at IAA concentrations of 0·1 mg./l. and above. It may be noted that IAA uptake is not a simple process of diffusion (Reinhold, 1954) but involves at least two components, one physical and one metabolic. Alternatively, if uptake was unaffected, then inhibitor may have been interfering in IAA-regulating growth processes. If this is true, then differences between Fig. 5A and B permit one to suggest that (i) IAA is not primarily responsible for the endogenous growth of wheat coleoptile sections in water, or (ii) sites of auxin action used by applied IAA are not the same as those used by native auxins of the coleoptile; (i) and (ii) may be argued depending upon whether a relatively rapid or slow inhibitor penetration to a growth sites is postulated. It may be noted that Audus (1954) has expressed a similar view to (i) for oat coleoptiles during a critical appraisal of the work of McRae and Bonner (1953), while opinions similar to (ii) have been recently made by Audus and Brownbridge (1957).

While various interpretations to account for Fig. 5A and 5B may be discussed, it is desirable to assess if possible the physiological significance of inhibitor-β in the control of growth processes. It has been seen that β from potato consists of a complex mixture of aliphatic acids while under some conditions an aromatic component, in this case scopoletin, could be present in the β zone of chromatograms too. The role of coumarins in growth and development is unknown although a wide literature exists on their examination. It is unknown whether β from wheat is similar to potato-extracted β, and opinions on a possible influence of β in cell elongation of coleoptiles are, therefore, of limited value here. Burton (1956) discovered a similar limitation in attempting to relate inhibitors in potato to potato dormancy using oat coleoptile sections as a bioassay.

A hormonal role for aliphatic acids in plant growth was discovered when English, Bonner, and Haagen-Smit (1939) isolated and characterized 1-decene-1:10-dicarboxylic acid from bean pods, a compound capable of inducing renewed cell division and cell extension activity in the parenchymatous cells of bean pod mesocarp. With views derived from earlier literature on 'wound hormones', they termed the substance 'traumatic acid'. During their investigations a large number of synthetic substances were subsequently tested, but activity was only found in certain homologues and analogues of traumatic acid. Of these, it is of interest to note that the hydrogenation product, decane-1:10-dicarboxylic acid, had approximately half the activity of traumatic acid while azelaic acid had slight activity. They concluded that two carboxylic acid groups in the molecule and a chain of 8 or more carbon atoms were essential for activity although subsequent work (Haagen-Smit and

Viglierchio, 1956) has indicated that the monocarboxylic acids, lauric, linolenic, and linoleic can be active too. From the above, it is conceivable that acid *A* could be active in this bean-division test.

Besides the bean test, another interesting result was cited by English *et al.* (loc. cit.). In a 'potato test' of Haberlandt, intense cell-division activity was evoked by traumatic acid and it was thought that the acid is probably responsible for wound periderm formation in the potato. It would appear that the β complex examined in the present work may be part of this system and the use of a potato-cell-division test might be appropriate for examining the physiological properties of β from potato.

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